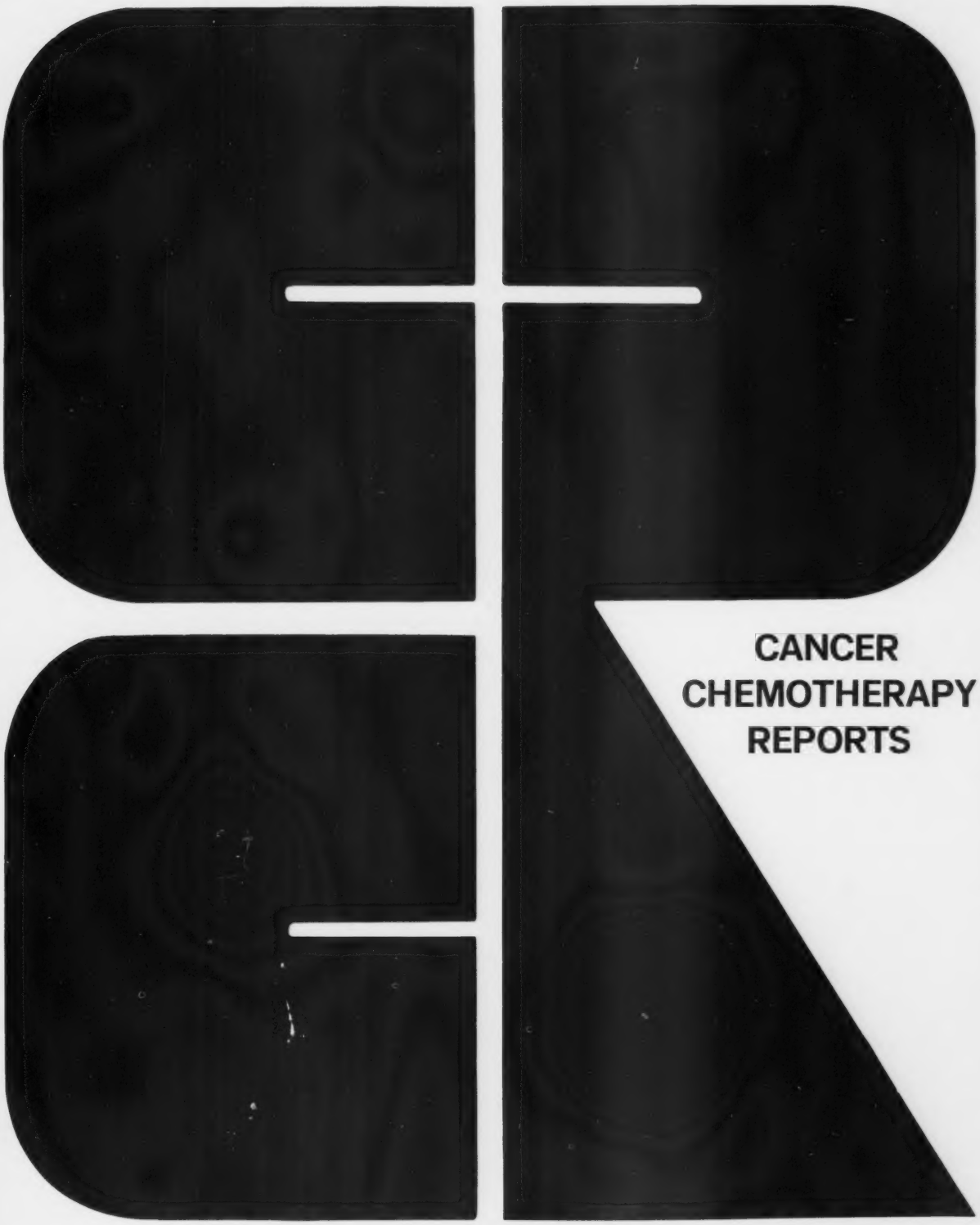

CANCER CHEMOTHERAPY REPORTS

Part 1

May/June 1975
Vol. 59, No. 3

Cancer Chemotherapy Reports

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Begin the article with a short summary.

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Submit an original and three copies of the manuscript, typed double spaced. Each page should begin with a new paragraph. Use accepted spelling, abbreviation, and hyphenation found in *Webster's New International Dictionary*. Footnotes should be typed at the bottom of the page on which they appear.

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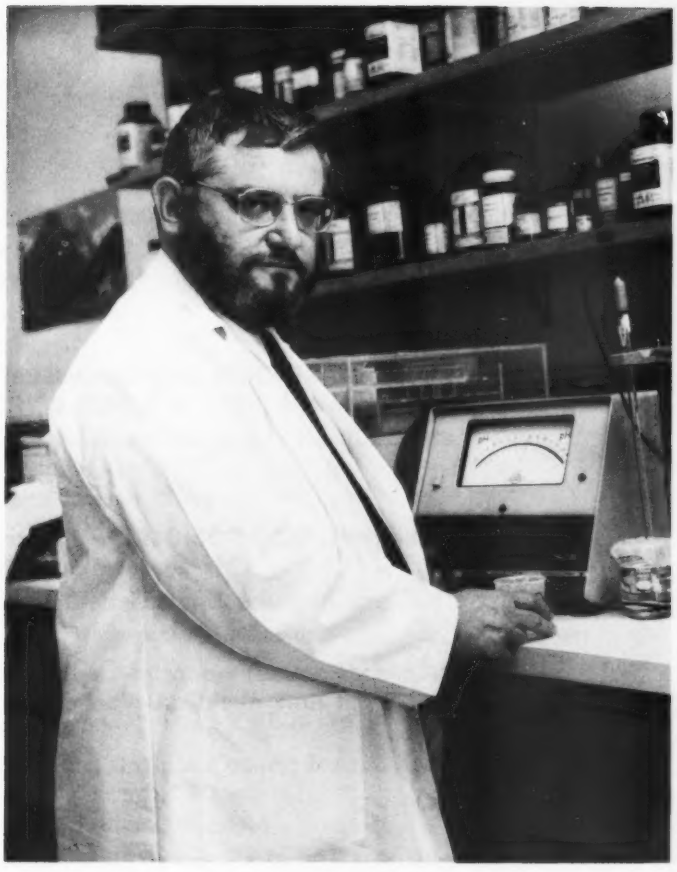

Myron R. Karon, MD, died recently in Los Angeles of a cerebral hemorrhage at the age of 42. At the time of his death he was a Professor of Pediatrics and the Cancer Coordinator for Pediatric Oncology at the USC School of Medicine; Head, Division of Hematology-Oncology at Children's Hospital of Los Angeles; and a Scholar of the Leukemia Society of America. He had previously served as a senior investigator in the Leukemia Service of the Medicine Branch, National Cancer Institute. An acknowledged pioneering leader in this country in the management of pediatric malignancy, an articulate and provocative thinker, a compassionate physician, Dr. Karon was at least these to those who knew him. Unlike other gifted investigators he was able to communicate his ideas to his students, clinical peers, and patients, and thereby developed a laboratory and clinical program of unusual excellence in oncology; his death removes a major force in the fight against cancer.

Friends of Myron remember him also as an accomplished musician, a tenacious tennis player, a person of moral strength and unusual sensitivity. He was beloved by many. At his funeral it was asked "How do we measure a man's life . . . by its length or by its use?"

He is survived by his wife Arlene and three sons. The Myron Karon Memorial Fund has been established at the Children's Hospital of Los Angeles.

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

LETTER

Combination Chemotherapy Using Agents With Limited Effectiveness

In a letter to *Cancer Chemotherapy Reports*, Bunn (1) asks himself: "Should ineffective agents be used in combination chemotherapy?" But Kovach et al (2), in the article that started the question, stated that "*cis*-diamminedichloroplatinum appears to have *little* value." Therefore, Bunn's question should really be: should agents with "little" effectiveness be used in combination chemotherapy?

In the fight against bacilli it has been proven that drugs aggregate or potentiate. For example, Smith and McClosky (3) demonstrated drug potentiation by combining Promin with streptomycin against the Koch bacillus, although Promin is a drug with limited effectiveness. If in the case of bacterial cells drugs aggregate or potentiate, couldn't something similar occur with regard to tumor cells? Tashima (4), Rosenberg (5), and Street (6) have noted a greater effectiveness when using combination therapy against the neoplastic process. We have established under bronchoscopic examination the disappearance of bronchial carcinoma by using a combination of drugs at their minimum effective doses (7). Each of these drugs used alone would have had very little or no effectiveness on the disease. With this type of combination we have also had a 39% survival over a 6-month period in very far-advanced bronchogenic carcinoma (8). Although we don't know the exact mechanism of action, such effects could be explained by drug potentiation and/or stimulation of the lymphocytes. Studies by Svet-Moldavsky et al (9,10), and also unpublished observations of ours, point in this direction.

Therefore, we agree in many respects with the opinion given by Tashima (4), Rosenberg (5), and Street (6) concerning the question raised by Bunn (1). The use of drug combinations is a necessity in the effort to control resistance. Combination therapy using the minimum effective dose seems to us a new and important approach. This approach would allow treatment with nontoxic, nonimmunodepressive doses. Very probably, these doses could also stimulate cellular immunity.

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February 11, 1975

NOTE

The Editors of *Cancer Chemotherapy Reports* would like to thank all those who have contributed to the discussion concerning combination chemotherapy using single ineffective agents. Although we are interested in additional opinions on this subject, we will not be including such letters in future issues of the journal.

Enhancement by Tetrahydrouridine (NSC-112907) of the Oral Activity of 5-Azacytidine (NSC-102816) in L1210 Leukemic Mice [1,2,3]

Gary L. Neil, Thomas E. Moxley, Sandra L. Kuentzel, Rita C. Manak, and Ladislav J. Hanka [4,5,6]

SUMMARY

5-Azacytidine is more active when administered parenterally than orally in the treatment of L1210 leukemic mice. Oral coadministration of tetrahydrouridine, a pyrimidine nucleoside deaminase inhibitor with no intrinsic antitumor activity, greatly increases the oral activity of 5-azacytidine. 5-Azacytidine (or cytotoxic equivalent) blood levels in BDF_1 mice are much higher after oral administration of the 5-azacytidine-tetrahydrouridine combination than when 5-azacytidine is administered alone by the same route. The therapeutic results (L1210 leukemia) achieved with the oral combination are similar to those observed with parenteral 5-azacytidine alone.

[Cancer Chemother Rep 59:459–465, 1975]

5-Azacytidine has shown antitumor activity against transplanted tumors (T-4 lymphoma [1]and L1210 leukemia [1,2]) and spontaneous tumors (AK lymphoma [3]) in mice. It is also an immunosuppressant (4) and an abortifacient (5) in this species. The biochemical mode of action of 5-azacytidine has been actively studied (4,6–9) and clinical trials of its effect in the treatment of human neoplasia are in progress (10–17). 5-Azacytidine has been shown to be an effective treatment of acute leukemia in children (12–14) including tumors resistant to another cytosine nucleoside antimetabolite, cytosine arabinoside (12,13).[7]

We have reported the effects of tetrahydrouridine, a potent inhibitor of pyrimidine nucleoside deaminase, on the oral activity of cytosine arabinoside (18). Although it possesses no antitumor activity itself, tetrahydrouridine markedly increases the oral activity (in L1210 leukemic mice) of cytosine arabinoside by modifying its metabolism and effective absorption. The effects of tetrahydrouridine on the antitumor activity of 5-azacytidine are reported here.

METHODS

The effects of 5-azacytidine and combinations of 5-azacytidine and tetrahydrouridine on the survival of L1210 leukemic mice were studied

[1] Received May 3, 1974; revised Jan 14, 1975; accepted Jan 22, 1975.

[2] This study was supported in part by contract PH43-68-1023 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Tetrahydrouridine: CAS reg. No. 5627-05-4; 2(1*H*)-pyrimidinone, tetrahydro-4-hydroxy-1-β-D-ribofuranosyl-; THU. Provided by Dr. Arthur R. Hanze (deceased) or Dr. Clifford Y. Peery of The Upjohn Company, Kalamazoo, Mich.

5-Azacytidine: CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-; 5-azaCR, Obtained from the Alfred Bader Chemical Company or through the courtesy of Dr. David G. Martin of The Upjohn Company.

[4] Cancer Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

[5] The authors wish to thank Lynda G. Gray for her technical assistance and Elizabeth L. Clark for assistance in the preparation of this manuscript.

[6] *Reprint requests to:* Gary L. Neil, PhD, Experimental Biology Research Department, Cancer Research, The Upjohn Company, Kalamazoo, Mich 49001.

[7] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytarabine; Cytosar; ara-C.

using previously described methods (18) following protocols described by Drug Research and Development, DCT, NCI (19). In all experiments, female BDF_1 (C57BL × DBA/2) mice weighing 18–20 g were used (eight animals/group). Agents were dissolved in sterile 0.9% saline and administered (intraperitoneal [ip] injection or oral intubation) in a constant total volume of 0.2 ml/mouse. Fresh solutions were prepared each day. Animals were inoculated ip on Day 0 with 1×10^6 L1210 cells/mouse and treatment was initiated the following day.

Previous studies (18) have shown that tetrahydrouridine has no intrinsic antitumor effect. Thus, control animals received only oral doses of saline. Where combinations were employed, half of the tetrahydrouridine dose (0.1-ml volume) was administered one-half hour before 5-azacytidine administration and the other half immediately prior to 5-azacytidine administration. Mean survivals, with standard deviations, were calculated for treated and control groups. Percent increases in lifespan (% ILS) were then calculated from these data. Each group of animals was weighed on Days 0 and 5 and the average weight change (g/mouse) was calculated.

Levels of 5-azacytidine (or cytotoxic equivalent) in BDF_1 mouse blood were determined after administration of 5-azacytidine in a dose of 50 mg/kg, ip (experiment 1) or orally (experiment 2) alone or in combination with tetrahydrouridine in a dose of 10 mg/kg by the same route. A divided tetrahydrouridine dose (*see* above) was used. Blood was obtained from the vena cava of anesthetized (Metofane, Pittman Moore Division, Dow Chemical Co., Southgate, Mich) mice (three/time) at various times after 5-azacytidine administration and was stored on ice for less than 3 hours until assay. Blood or a 1:5 dilution in 0.9% saline from each mouse was assayed separately.

The assay employed was a modification of the microbiologic disc-plate assay reported by Haňka et al (1). The assay organism was *Escherichia coli* (ATCC-26) growing in a fully synthetic medium. The synthetic medium consisted of a broth containing KH_2PO_4, 3.0 g; K_2HPO_4, 7.0 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 1.0 g; glucose, 2 g; tetrahydrouridine, 50 mg; and distilled water to make 1 liter, supplemented with 1.5% agar (high-grade granulated agar, BBL, Cockeysville, Md). Subsequent to autoclaving, the glucose and tetrahydrouridine [8] were added. The seed consisted of 1.0 ml of culture (16–18 hours)/500 ml of agar. Aliquots of 100 ml each were then dispensed into plastic trays (20 cm × 50 cm) (20).

Standard solutions were prepared by dissolving 5-azacytidine (0.5–16 μg/ml) in whole mouse blood (heparinized) or mouse blood diluted (1:5) with 0.9% saline. Standard solutions, in triplicate, and test samples, full-strength blood and 1:5 dilutions in saline in duplicate, were applied to 12.7-mm diameter paper discs (Carl Schleicher and Schuell Co., Keene, NH) on the seeded agar trays. After preincubation at 4° C for 2 hours (which was found to improve sensitivity) trays were incubated overnight at 32° C. Diameters of zones of inhibition were then measured.

Typical standard curves are shown in figure 1. Standard curves were constructed using linear regression analysis on individual assay values; however, only mean values are shown. The sensitivity limits in experiments 1 and 2 were 1.0 and 0.5 μg of 5-azacytidine (or equivalent)/ml of mouse blood, respectively. The concentrations of 5-azacytidine in the test samples were determined using the appropriate standard curve.

The sensitivity of the assay organism to a number of available metabolites or degradation products of 5-azacytidine was also determined. 5-Azacytosine [9] (VI, fig 2), ribose (VIII, fig 2), guanidine (V, fig 2), and biuret (XIII, fig 2)

[8] Previous experiments not described here showed that the addition of tetrahydrouridine (50 μg/ml) to the assay medium increased sensitivity from two- to four-fold, depending on the diluent employed. Similar results have been observed with the sensitivity of *Streptococcus faecalis* (actinobolin-resistant) to cytosine arabinoside (20). No additional increase in sensitivity was observed with concentrations of 100 or 200 μg/ml of tetrahydrouridine. In addition, levels of tetrahydrouridine in blood samples themselves (up to 50 μg/ml) had no effect on the 5-azacytidine values observed.

[9] 5-Azacytosine and 5-azauracil were purchased from Calbiochem, Los Angeles, Calif, and Nutritional Biochemicals Corporation, Cleveland, Ohio, respectively.

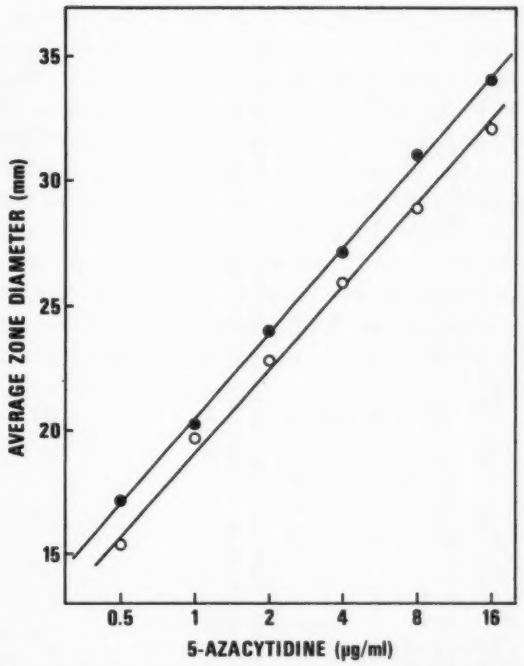

FIGURE 1.—Standard curve for inhibition of *E. coli* by 5-azacytidine. ○ = solutions in mouse blood; ● = solutions in mouse blood diluted (1:5) with 0.9% saline. Pearson's correlation coefficients (r) were 0.9995 and 0.9980 for the mouse blood curve and the diluted blood curve, respectively.

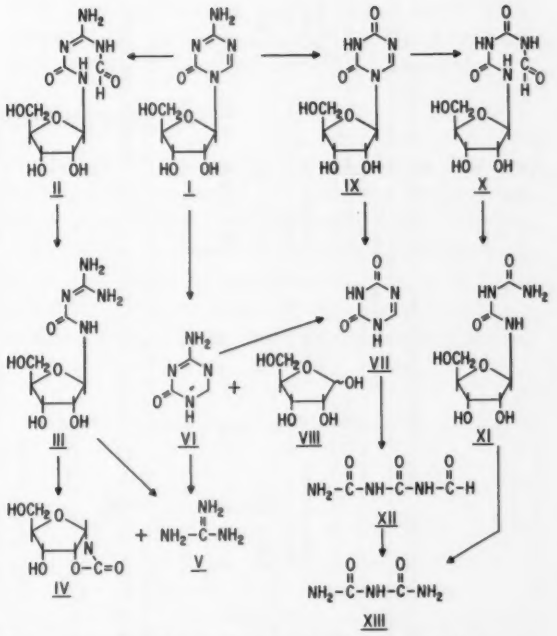

FIGURE 2.—Possible pathways for the degradative metabolism of 5-azacytidine. I = 5-Azacytidine; II = 1-ribofuranosyl-3-(formylguanyl)urea (unstable); III = 1-ribofuranosyl-3-guanylurea; IV = α-D-ribofuro[1′,2′:4,5]-2-oxazolidone; V = guanidine; VI = 5-azacytosine; VII = 5-azauracil; VIII = ribose; IX = 5-azauridine (unstable); X = ribofuranosylformyl biuret; XI = ribofuranosylbiuret; XII = 1-formylbiuret; XIII = biuret.

were completely inactive at concentrations of 1 mg/ml. 5-Azauracil [9] (VII, fig 2) had approximately one seventh the activity of 5-azacytidine. Thus, all data are expressed as "5-azacytidine (*or* cytotoxic equivalent)."

RESULTS

The effects of tetrahydrouridine on 5-azacytidine therapy of L1210 leukemic mice (single-dose and daily dose schedules) are shown in tables 1 and 2, respectively. 5-Azacytidine used alone was much more effective (especially on a daily dose schedule) when administered ip than when administered orally. For example, daily treatment with 2.5 mg/kg of 5-azacytidine ip was considerably more effective (150% ILS) than treatment with an oral dose of 10 mg/kg/day (83% ILS) (table 2). Using both schedules, ip tetrahydrouridine had little effect, if any, on ip therapy with 5-azacytidine. However, tetrahydrouridine significantly *increased* the effectiveness of 5-azacytidine when both agents were administered orally. Oral therapy (both schedules) with this combination was approximately as effective as therapy with ip 5-azacytidine used alone. Toxicity, as measured by early mortality and weight loss, was also increased when tetrahydrouridine was employed in an oral combination with 5-azacytidine. In figure 3, mean survival is shown as a function of weight change. The weight loss observed at a particular ILS appeared to be independent of the use of tetrahydrouridine. Thus, the therapeutic ratio with the combination does not appear to differ significantly from that observed with 5-azacytidine used alone.

TABLE 1.—Therapy of L1210 leukemic mice with combinations of 5-azacytidine and tetrahydrouridine—single-dose therapy*

5-Azacytidine dose (mg/kg)	Ip administration of—						Oral administration of—					
	5-Azacytidine			5-Azacytidine + tetrahydrouridine			5-Azacytidine			5-Azacytidine + tetrahydrouridine		
	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)
0 (control)	6.9 ± 0.4		+2.3	6.9 ± 0.4		+2.3	6.9 ± 0.4		+2.3	6.9 ± 0.4		+2.3
12.5	9.8 ± 0.6	42	−0.4	11.3 ± 1.1	64	−1.7	8.0 ± 0.7	16	+1.4	10.6 ± 0.9	54	−1.1
50.0	12.7 ± 0.4	84	−2.3	12.6 ± 0.4	83	−2.8	9.9 ± 0.9	43	−1.0	13.9 ± 1.4	100	−3.4

*Mice were inoculated on Day 0 with 1×10^5 L1210 ascites cells; therapy was started on Day 1; tetrahydrouridine (10 mg/kg) was administered in a divided dose (*see* Methods) by the same route as employed for 5-azacytidine. Control animals received only saline orally. Results are expressed as mean survival times (± SD) and % ILS calculated therefrom.

†Animals were weighed on Days 0 and 5.

TABLE 2.—Therapy of L1210 leukemic mice with combinations of 5-azacytidine and tetrahydrouridine—daily therapy*

5-Azacytidine dose (mg/kg)	Ip administration of—						Oral administration of—					
	5-Azacytidine			5-Azacytidine + tetrahydrouridine			5-Azacytidine			5-Azacytidine + tetrahydrouridine		
	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)	Mean survival (days) ± SD	% ILS	Weight change† (g/mouse)
0 (control)	7.1 ± 0.6		+2.2	7.1 ± 0.6		+2.2	7.1 ± 0.6		+2.2	7.1 ± 0.6		+2.2
2.5	17.8 ± 1.6	150	−3.5	20.3 ± 3.1	186	−4.7	8.6 ± 1.2	21	−0.2	16.2 ± 4.0	128	−4.3
5.0	19.3 ± 2.0	172	−4.1	19.4 ± 1.6	173‡	−4.9	9.6 ± 0.8	35	−2.3	22.7 ± 2.5	220§	−4.7
10.0	19.0 ± 0.8	168‡	−4.7	5.1 ± 1.3	Toxic	−4.7	13.0 ± 1.7	83	−3.6	8.3 ± 2.4	Toxic	−4.9

*Mice were inoculated on Day 0 with 1×10^5 L1210 ascites cells; therapy was started on Day 1 and continued daily for 4 days; tetrahydrouridine (10 mg/kg/day) was administered in a divided dose (*see* Methods) by the same route as employed for 5-azacytidine. Control animals received only saline orally. Results are expressed as mean survival times (± SD) and % ILS calculated therefrom.

†Animals were weighed on Days 0 and 5.

‡Two toxic deaths not included in calculation of mean survival times.

§1/8 Long-term (45-day) survivor.

The effects of tetrahydrouridine on 5-azacytidine mouse blood levels after ip or oral administration of the combination are shown in figure 4. Levels are compared to those found after administration of 5-azacytidine alone. After oral administration of 50 mg/kg of 5-azacytidine alone, maximum 5-azacytidine blood levels were much lower (about 2.1 μg/ml) than after a similar ip dose (about 43 μg/ml). Coadministration of tetrahydrouridine resulted in little increase of maximum 5-azacytidine levels when both agents were administered ip. However, maximum levels were increased approximately three times (to about 6.2 μg/ml) when an oral combination was employed. With both routes, the time during which levels of 5-azacytidine could be detected in blood was increased when tetrahydrouridine was used.

DISCUSSION

The effects of tetrahydrouridine on the oral antileukemic activity of cytosine arabinoside have been extensively discussed (18). The results of the present study with tetrahydrouridine and another cytosine nucleoside antimetabolite, 5-azacytidine, are very similar to those of the previous study with cytosine arabinoside.

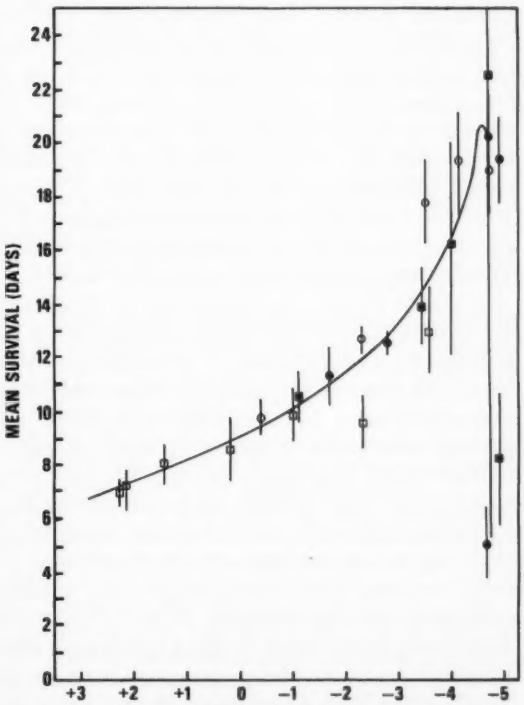

FIGURE 3.—Therapeutic ratio with 5-azacytidine and 5-azacytidine + tetrahydrouridine combinations in the treatment of L1210 leukemic mice. Survival of L1210 leukemic mice (mean ± SD) is shown as a function of weight change (average g/mouse, Days 0–5) observed with various treatments (routes and schedules) with 5-azacytidine or 5-azacytidine + tetrahydrouridine combinations. Data are composite and were obtained from tables 1 and 2. ● = Ip 5-azacytidine + tetrahydrouridine; ○ = ip 5-azacytidine; ■ = oral 5-azacytidine + tetrahydrouridine; □ = oral 5-azacytidine.

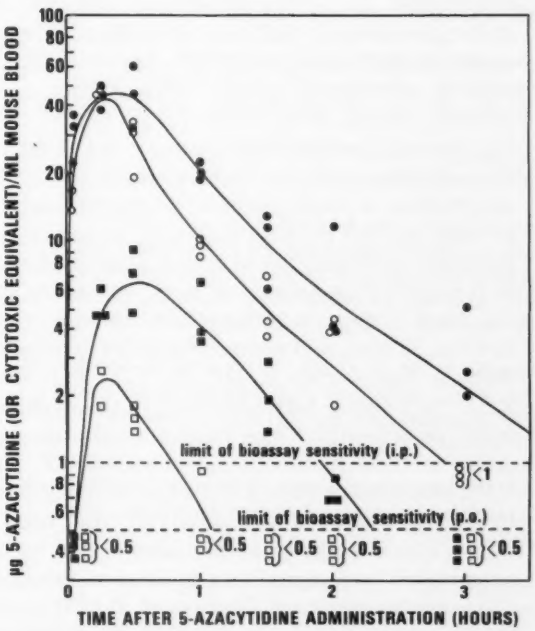
μg 5-AZACYTIDINE (OR CYTOTOXIC EQUIVALENT)/ML MOUSE BLOOD
limit of bioassay sensitivity (i.p.)
limit of bioassay sensitivity (p.o.)
TIME AFTER 5-AZACYTIDINE ADMINISTRATION (HOURS)

FIGURE 4.—Blood levels of mice receiving 5-azacytidine (or cytotoxic equivalent) after a single administration of 5-azacytidine or a 5-azacytidine + tetrahydrouridine combination. 5-Azacytidine (50 mg/kg) was administered (ip or orally) alone or in combination with tetrahydrouridine (10 mg/kg by the same route). Blood from three mice/time was obtained 5, 15, 30, 60, 90, 120, and 180 min after 5-azacytidine administration. Blood levels were determined with a microbiologic disc-plate assay employing *E. coli* growing in a fully synthetic medium supplemented with tetrahydrouridine (50 μg/ml). ● = Ip 5-azacytidine + tetrahydrouridine; ○ = ip 5-azacytidine; ■ = oral 5-azacytidine + tetrahydrouridine; □ = oral 5-azacytidine.

In both cases, the oral activity of the antitumor agent, administered alone, is much less than that observed parenterally. With both agents, this oral activity is markedly increased by concomitant oral administration of the deaminase inhibitor. Also, in both cases, coadministration of tetrahydrouridine resulted in increased blood levels of the antitumor agent (or cytotoxic equivalent) after oral administration.

In the case of cytosine arabinoside, enzymatic deamination is certainly implicated as a major reason for the low oral activity observed, and the inhibition of this deamination by tetrahydrouridine is apparently the reason for the observed increase in oral activity (18). With 5-azacytidine, it is somewhat more difficult to state unequivocally that this is the case. Whereas the catabolic metabolism of cytosine arabinoside is exquisitely simple (the deamination product, uracil arabinoside [NSC–68928] being the only metabolite observed), the metabolism, including nonenzymatic degradation, of 5-azacytidine is very complex. A representation of some of the possible degradative pathways involved in 5-azacytidine metabolism is shown in figure 2. This scheme is based primarily on the work of Sorm's group (4,21–24) involving in vitro, in vivo, and nonenzymatic (hydrolysis) studies. Compounds I, III, V, VII, XII, and XIII (fig 2) have been identified in the urine of mice treated with 5-azacytidine (4,21). In addition to these metabolites, compounds IV, VI, VIII, and, transiently, II (fig 2) arise in aqueous hydrolysis studies under relatively mild conditions (23). 5-Azacytidine has been shown to be deaminated to 5-azauridine by *Escherichia coli* (24). Recent results of Chabner et al (25) show that 5-azacytidine is deaminated by a human leukemic cell deaminase (the same enzyme which deaminates cytosine arabinoside) and that tetrahydrouridine inhibits this deamination.[10] 5-Azauridine (IX, fig 2) appears (13,14) to be even less stable to hydrolysis than 5-azacytidine and thus it may not be too surprising that it was not observed in mouse urine. Further degradation products of the 5-azauracil moiety (XII and XIII, fig 2) were found however (21). Although these products could arise via pathways (eg, I → VI → VIII, fig 2) other than those involving deamination of 5-azacytidine to 5-azauridine (I → IX, fig 2), this latter route is certainly probable. Indeed the postulated conversion of 5-azacytidine (I, fig 2) to 5-azacytosine (VI, fig 2) is rather unlikely since it might be expected that, like cytidine, it would not be a substrate for any of the known mammalian phosphorylases (26).

Although the deamination product of cytosine arabinoside (uracil arabinoside) has shown no biologic activity and, in particular, does not increase the lifespan of L1210 leukemic mice (unpublished results, these laboratories), the biologic effects of the 5-azacytidine metabolites are less clearly understood. 5-Azauracil (VI, fig 2) has shown some antitumor activity (27) although it has been reported to be inactive in L1210 leukemia. Although no data are available concerning their antitumor activities, 5-azauridine itself and *N*-formylbiuret (compound XII, fig 2) have been shown to possess some, but relatively low, antibacterial activity (28,29).

The lack of any significant effect of tetrahydrouridine on the therapeutic activity of ip administered 5-azacytidine is reasonably consistent with the rather marginal effects of tetrahydrouridine on 5-azacytidine blood levels (especially maximum levels) after administration by that route. Similarly, the enhancement of 5-azacytidine's oral activity by tetrahydrouridine correlates well with the enhanced blood levels observed. However, although the antitumor activity of the oral combination was roughly equivalent to that observed with ip 5-azacytidine alone, blood levels in the former case were clearly inferior to those observed with ip administration. This discrepancy is not easily explained. In our studies, blood was the only tissue assayed for 5-azacytidine activity. Perhaps studies of the kinetics of the disposition of 5-azacytidine in other tissues (particularly lymphoid tissues, ascites fluid, and the tumor itself) would yield more meaningful data for such correlations.

Further studies aimed at determining the importance of nucleoside deamination and its inhibition by tetrahydrouridine on the disposition,

[10] Indeed, of the reactions illustrated in figure 2, the deamination of 5-azacytidine to 5-azauridine is the only one which has definitively been demonstrated to proceed enzymatically.

metabolism, and biologic activity of 5-azacytidine are in progress in our laboratories.

In conclusion, tetrahydrouridine has been shown to increase the effectiveness of another antitumor cytosine nucleoside antimetabolite, 5-azacytidine. The possible clinical use of this inhibitor in combination with agents such as cytosine arabinoside and 5-azacytidine would seem to warrant careful consideration.

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Cis-dichlorodiammineplatinum (II) (NSC-119875): Hearing Loss and Other Toxic Effects in Rhesus Monkeys [1,2,3,4]

S. W. Stadnicki, R. W. Fleischman, U. Schaeppi, and P. Merriam [5,6,7]

SUMMARY

Ototoxicity and general toxicity of *cis*-dichlorodiammineplatinum (II), a compound with antitumor activity, were evaluated in four rhesus monkeys. Audiometry was performed with shock avoidance to conditioned stimuli of pure tones at frequencies from 0.25 to 18 kHz. Histopathologic lesions of the inner ear were identified with phase-contrast microscopy of surface preparations from the organ of Corti. The monkeys were also submitted to physical examinations, hematologic and blood chemical determinations, necropsies, and histopathologic evaluations. *Cis*-dichlorodiammineplatinum (II) was injected intravenously in one monkey in five daily doses of 1.25 mg/kg (LD50) and in three monkeys in ten to 26 doses of 0.625 mg/kg five times per week. Transient hearing loss occurred in the monkey treated with the largest dose and in one of the three animals treated at the lower dose. In both monkeys apparently normal hearing reappeared after 120 and 200 days. No hearing loss was detectable in the other two animals. All four monkeys, however, had loss of hair cells in the organ of Corti and lesions were most severe among the outer hair cells of the lower turns. Other effects in all four monkeys included severe chronic glomerulonephritis with intermittent azotemia. Two monkeys (positive controls) injected intramuscularly with ten or 25 daily doses of neomycin sulfate (50 mg/kg) responded with delayed permanent hearing loss that appeared 3 months after the start of treatment. Deafness was substantiated by a total loss of inner hair cells in the upper turns, by a severe loss in the lower turns, and by a moderate or severe loss of outer hair cells in the lower turns. The above observations indicated that *cis*-dichlorodiammineplatinum (II), at a treatment regimen which caused more severe organ toxicity, caused less severe ototoxicity than neomycin sulfate and destroyed hair cells at different sites than neomycin sulfate.

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[2] Supported in part by contract NIH-70-2055 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] *Cis*-dichlorodiammineplatinum (II): CAS reg. No. 15663-27-1. Supplied by Drug Research and Development, DCT, NCI.

[4] This paper is based on portions of a dissertation submitted by the first author in partial fulfillment of the requirements for the PhD degree in the Biomedical Engineering Program, Department of Life Sciences, Worcester Polytechnic Institute, Worcester, Mass. In addition, the paper summarizes observations that were included in the following detailed report submitted to the Laboratory of Toxicology, Experimental Therapeutics, DCT, NCI (*see also* reference 12 and footnotes 8,

Cis-dichlorodiammineplatinum (II), a compound with antimitotic activity (1,2), has potent antineoplastic activity against various experimental tumor systems (3–5). Patients treated with large doses of the drug exhibited gastrointestinal toxicity (nausea and emesis), bone marrow depression, renal tubular necrosis (azotemia), tinnitus, and transient or permanent hearing loss at high-frequency tones (6–9). Ototoxicity was not noted in preclinical studies with *cis*-dichlorodiammineplatinum (II) (10–12),[8] but this effect is known to occur after treatment with anticancer agents such as mechlorethamine (13), bleomycin (14), and 6-aminonicotinamide (15).[9] The purpose of this study was to investigate the ototoxicity of *cis*-dichlorodiammineplatinum (II) in rhesus monkeys, to relate it to other toxicity, and to compare it with the effects of neomycin sulfate, a known ototoxic antibiotic.

METHODS

Experiments were performed on eight rhesus monkeys (Macaca mulatta) of both sexes with a body weight ranging from 2.7 to 4.3 kg and with an estimated age of 3–5 years. For audiometry they were restrained in a primate chair with the head rigidly immobilized to prevent movement. The monkeys were tested in a sound-attenuated chamber with the equipment located outside. During testing, the animals were observed through a one-way glass window.

The animals were conditioned to respond to a pure tone by pressing a lever. Failure to press the lever was "punished" with an electric tail shock. The initial conditioned stimulus was a pure tone of 1 kHz, at 60 decibels, calibrated re. 0.0002 dyne/cm^2. At the beginning of conditioning, some animals continuously depressed the lever, but this response was overcome by additional shocks until the monkeys learned to release the lever after each response. In conditioned monkeys, electric shocks were delivered with a Lehigh Valley automated shocker-timer system (shocker 113-02, interval timer 253-11, and power supply 215-04). If monkeys failed to respond to the tone, electric shocks were given 2 seconds later and were usually continued until the monkey pressed the lever. Tail shock (50 Hz, 0.1–5 ma with 10 kohms of output resistance) was applied via platinum subdermal electrodes (type E2, Grass Instruments). Monkeys that were poor performers were not used in this study.

Audiometry was performed during and after treatment once per week at frequencies of 1, 8, and 18 kHz, and once per month at frequencies of 0.125, 0.250, 0.5, 2, 4, 12, and 16 kHz. Testing at high frequencies of 12–18 kHz was introduced later during the study. Tones were presented for a duration of approximately 1 second. At the beginning of each testing session the hearing threshold was roughly estimated. Then, for a range of 20 decibels of the estimated threshold, pure tones were presented at decreasing sound pressure levels of 5 decibels. For each sound pressure in this 20-decibel range the tone was presented six times. The auditory threshold was defined as that sound pressure level which produced 50% or more correct responses. Frequencies of 125–8000 Hz were supplied with an Eckstein Brothers 350-1 audiometer (calibrated re. 0.0002 dyne/cm^2) and presented via an aural dome head set. Frequen-

11, and 12): Schaeppi U, Fleischman RW, Stadnicki SW, et al. *Cis*-dichlorodiammine platinum (II) (NSC-119 875): ototoxicity in rhesus monkeys. Final report to the Laboratory of Toxicology, Experimental Therapeutics, DCT, NCI, Dec 31, 1973 (MRI-CC14-73-52).

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[6] We thank our collaborators from Mason Research Institute, Dr. V. Ilievski for veterinary services; Dr. H. Rosenkrantz and Mr. Y. Luthra for supervision of the hematology and clinical chemistry tests; and Mr. R. Rosetti, Mr. A. Estaphan, and Mr. M. Ethier for the audiometry of rhesus monkeys.

[7] *Reprint requests to:* Stanley W. Stadnicki, MA, Mason Research Institute, 23 Harvard St, Worcester, Mass 01608.

[8] Heyman IA, Schaeppi U, Fleischman RW, et al. *Cis*-dichlorodiammine platinum (II) (NSC-119 875): comparison of urinary lactic dehydrogenase and alkaline phosphatase levels with serum BUN levels as indicator of renal toxicity in rhesus monkeys. Formal report to the Laboratory of Toxicology, Experimental Therapeutics, DCT, NCI, Sept 8, 1972 (MRI-PLAT-03-72-37).

[9] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

Bleomycin: NSC-125066.

6-Aminonicotinamide: NSC-21206; CAS reg. No. 329-89-5.

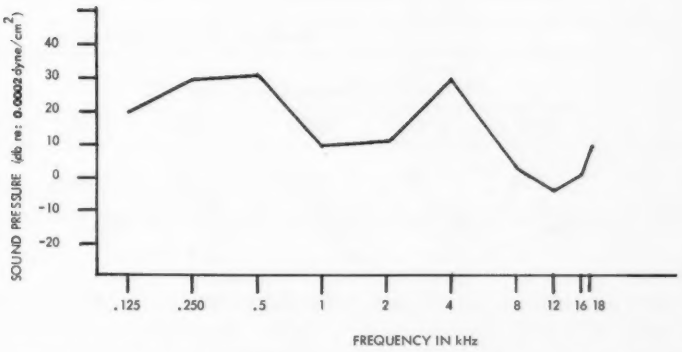

FIGURE 1.—Standard audiogram representing typical hearing threshold levels for rhesus monkeys used in ototoxicity studies. Monkey No. 413, right ear.

cies of 12, 16, and 18 kHz were produced with a Tracor RA114 HF audiometer (calibrated re. 0.0002 dyne/cm²) and presented via a cone positioned in contact with the external auditory meatus. Control audiograms were performed for both ears, whereas routine tests were restricted to the right ear. Figure 1 depicts a standard audiogram for control monkey No. 413.

Surface preparations of the organ of Corti were prepared according to the methods of Johnsson and Hawkins (16) and Engström et al (17) (fig 2). Cochleograms, which are exact cell-for-cell diagrams of the pathology of the organ of Corti, were made from representative segments of the hook, and from turns ½, 1½, 2½, and 3 of the cochlea. Figure 3 shows a standard cochleogram from untreated control monkey No. 413.

Blood determinations, clinical observations, and gross and histopathologic evaluations were carried out as described by Schaeppi et al (12).[10] Experiments were performed with *cis*-dichlorodiammineplatinum (II) and neomycin sulfate (purchased from Sigma Chemical Co.).

Cis-dichlorodiammineplatinum (II) was injected intravenously (iv) in two monkeys in five daily doses of 1.25 mg/kg (LD50) and in three monkeys in ten to 26 doses of 0.625 mg/kg five times per week. The two positive control monkeys were injected intramuscularly (im) with ten or 25 daily doses of neomycin sulfate (50 mg/kg) (table 1). One untreated control monkey was submitted to audiometry throughout the entire study.

[10] *See* footnote 8.

RESULTS

Ototoxicity is described as it occurred during the course of other toxic manifestations. Treatment of two rhesus monkeys (Nos. 377 and 398) with *cis*-dichlorodiammineplatinum (II) at doses of 1.25 mg/kg for 5 consecutive days caused death in one monkey and severe toxicity in the other. Monkey No. 377 died on Day 13. Blood determinations revealed uremia (blood urea nitrogen [BUN] level, 181 mg/100 ml) and serum electrolyte derangements (Na^+, 138 mEq/liter; K^+, 7.5 mEq/liter). Pathologic findings included pulmonary edema and marked renal tubular dysplasia. Audiometry and histopathologic evaluation of the inner ear were not performed on this animal (table 1). Monkey No. 398 developed a reversible uremia with maximum changes from 6 to 18 days after the beginning of treatment. During uremia, the animal had a BUN of 183 mg/100 ml, a Na^+ of 158 mEq/liter, a K^+ of 3.2 mEq/liter, a Cl^- of 101 mEq/liter, a Ca^{++} of 8.1 mg/100 ml, and a relative metabolic acidosis with a pH of 7.2. Uremia regressed but thereafter the animal had a consistent mild azotemia (BUN, 20–30 mg/100 ml). The animal was necropsied in apparently good general condition 486 days after the beginning

TABLE 1.—Toxicity after repeated iv injections of *cis*-dichlorodiammineplatinum (II) or im injections of neomycin sulfate in rhesus monkeys*

	Monkeys (No.) treated with—						
	Cis-dichlorodiammineplatinum (II)					Neomycin sulfate	
	398F	377M	405M	410F	484F	397M	451M
Clinical observations							
Daily dose (mg/kg)	1.25	1.25	0.625	0.625	0.625	50	50
No. of doses	5	5	10	26	15	25	25
General condition	Poor	Died	Fair	Fair	Fair	Fair	Good
Emesis			+	+++	+		
Diarrhea				++	+		
Weight loss (%)	21	12	22	20	8	23	9
Blood determinations†							
Hemoglobin (g/100 ml)	9.9	10.1	8.2	6.7	7.9		10.2
Neutrophil count (cells/mm³)	1890	3385	2958	1135	947		
Lymphocyte count (cells/mm³)	1340	2600	1891	1222	1339		
Thrombocyte count (cells × 10^3/mm³)	130	180		170			
BUN (mg/100 ml)	183	181	91.9	53	92.5	55.4	30.9
Na^+ (mEq/liter)	158	138	142	140	123	155	
K^+ (mEq/liter)	3.2	7.5	3.5	3.4	5.7	3.1	
Cl^- (mEq/liter)	101	NT	98	99	82	91	
Ca^{++} (mg/100 ml)	8.1§	8.9‡			6.3‡	5.6	
pH	7.20	NT		7.28		7.533	
CO_2 (mEq/liter)	18.5	19.2				45	
SGOT (Reit Fränkel units/ml)	143		70	62		120	
SGPT (Reit Fränkel units/ml)	113			97		64	
BSP retention (%)	33.6		31.6	23.5	98	13.1	
Pathology							
Day of necropsy	486	13	411	443	247	415	225
General condition at necropsy	Good	Died	Good	Good	Good	Good	Good
Renal tubular metaplasia		++§					
Glomerulonephritis	++		++	++	++	+	
Prostatic atrophy and prostatitis						+	
Testicular degeneration						+	
Hearing loss at—¶							
1 kHz	++++R	NT	++++R				+++
8 kHz	++++R	NT	++++R			++++	+++
18 kHz	R	NT	R			++++	++++

*Rating of changes: + = moderate; ++ = marked; +++ = severe; ++++ = very severe; no entry = no toxicity; NT = not tested.

†Pretreatment values:

	Mean ± SD
Hemoglobin (g/100 ml)	12.5 ± 1.1
Neutrophil count (cells/mm³)	3890 ± 1690
Lymphocyte count (cells/mm³)	5298 ± 3000
Thrombocyte count (cells × 10^3/mm³)	35.5 ± 177
BUN (mg/100 ml)	18 ± 4.4

	Mean ± SD
Na^+ (mEq/liter)	147 ± 4.0
K^+ (mEq/liter)	4.5 ± 0.6
Cl^- (mEq/liter)	110.9 ± 4.5
Ca^{++} (mg/100 ml)	9.8 ± 0.6
pH	7.381 ± 0.075
Co_2 (mEq/liter)	28.2 ± 3.4
SGOT (Reit Fränkel units/ml)	40.2 ± 12.4
SGPT (Reit Fränkel units/ml)	28.9 ±12.8
BSP retention (%)	4.1 ± 3.5

‡Hypocalcemia followed by hypercalcemia.

§Acute toxicity also included hypocellularity of bone marrow and lymphoid tissues, atrophy of the pancreas, and edema of the lungs.

¶R = reversible hearing loss.

of treatment. Histopathologic lesions included a marked subacute glomerulonephritis. Moderate numbers of glomeruli were sclerosed and others showed marked periglomerular fibrosis. In addition, there were numerous interstitial aggregates of mononuclear cells in the cortex. On Day 128 temporary hearing loss for pure tones of 0.25–8 kHz occurred. The hearing loss persisted for frequencies of 4–8 kHz until Day 135. Thereafter, hearing recovered completely with normal hearing thresholds for 1, 8, and 18 kHz (fig 4). The left cochleogram showed a severe loss of outer hair cells, a marked loss of inner hair cells in the basal turn, and a moderate loss of hair cells in the other turns (fig 5). The right cochleogram showed a marked loss of outer hair cells in the basal turn, a moderate loss of outer hair cells in the other turns, and an occasional loss of inner hair cells (table 2). Figure 6 illustrates loss of outer hair cells.

In an effort to correlate ototoxicity with general toxicity, three monkeys (Nos. 405, 410, and 484) were treated with several courses of five repeated daily consecutive doses of 0.625 mg/kg until signs of renal toxicity with elevation in the BUN level (50–100 mg/100 ml) and derangements of serum electrolytes occurred. Other toxic effects included emesis in three of three monkeys; diarrhea, weight loss, moderate neutropenia, and lymphopenia in two of three monkeys; and anemia in three of three monkeys (table 1). In general, azotemia with a BUN level of from 30 to 50 mg/100 ml persisted intermittently throughout the observation period of 247–443 days. The three monkeys were killed in apparently good general condition but necropsies revealed a subacute glomerulonephritis in all three. Audiometry in one of the three monkeys (No. 405) showed a transient hearing loss for pure tones of 1 and 8 kHz (18 kHz was not tested). Hearing loss appeared on Day 17 (ie, 2 days after maximum elevation of BUN to 91.9 mg/100 ml), progressed on Day 40 to total transient deafness for pure tones of 1 and 8 kHz, persisted for approximately 70 days, and disappeared thereafter (fig 7). The other two monkeys (Nos. 410 and 484) had no hearing loss in spite of azotemia as high as 55 and 92 mg/100 ml respectively. The three monkeys treated at the lower dose exhibited a moderate or marked loss of outer hair cells in the lower turn, a moderate loss of outer hair cells in the other turns, and an occasional loss of inner hair cells (table 2).

Two positive control monkeys were injected im with consecutive daily doses of neomycin sulfate (50 mg/kg). Monkey No. 397, treated with 25 doses, exhibited anorexia, dehydration, muscle fibrillations, and weight loss. After termination of treatment on Day 26, the BUN level reached a peak of 55.4 mg/100 ml and serum derangements included K^+ (3.1 mEq/liter), Cl^- (91 mEq/liter), Ca^{++} (5.6 mg/100 ml), and pH 7.533. The serum glutamic oxaloacetic transaminase level had risen to 120 Reit Fränkel units and the serum glutamic pyruvic transaminase level had risen to 64 Reit Fränkel units. All values returned to normal limits after Day 73. Four to five weeks after the start of treatment, the monkey exhibited transient deafness at frequencies of 0.25, 0.5, and 8 kHz which disappeared entirely after Day 88. A second hearing loss appeared on Day 110, progressed on Day 169 to complete deafness for pure tones of 8 and 18 kHz, and persisted thereafter, while hearing at 1 kHz was not impaired (fig 8). The monkey was killed on Day 415 in apparently good general condition. Surface preparations from both

TABLE 2.—Missing hair cells in monkeys treated with *cis*-dichlorodiammineplatinum (II) or neomycin sulfate

Treatment	Monkey No.	Dose (mg/kg)	No. of treatments	Cochleogram*	Hair cells (%) missing in turn—†									
					3		2½		1½		½		Hook	
					I	O	I	O	I	O	I	O	I	O
Cis-dichlorodiammine-platinum (II)	398	1.25	5	L	0	0	8	8	0	3	24	53	—	—
				R	0	9	3	7	0	5	0	30	—	—
	405	0.625	10	R	0	8	0	7	0	5	0	47	—	—
	410	0.625	26	L	0	3	0	3	0	3	0	10	—	—
				R	3	0	0	5	5	0	0	15	3	21
	484	0.625	15	L	—	—	5	1	0	21	0	7	—	—
				R	—	—	3	0	0	5	0	3	0	0
				Mean	0.6	4.0	2.7	4.4	0.7	6.0	3.4	23.6	1.5	10.5
Neomycin sulfate	397	50	25	L	100	4	81	1	24	10	—	—	—	—
				R	100	—	100	6	8	20	—	—	—	—
	451	50	25	L	100	11	100	56	35	100	—	—	—	—
				R	100	0	100	20	100	83	—	—	—	—
				Mean	100	5	95	20.8	41.8	53.2				
Untreated control	413	0	0	L	0	4	0	2	0	4	8	0	0	0
				R	0	6	0	10.7	0	2	0	0	0	0
				Mean	0	5	0	6.4	0	3	4	0	0	0

*L = left ear; R = right ear.
†I = inner row; O = outer row; — = not available for evaluation.

inner ears showed a complete loss of inner hair cells in the upper turn and moderate loss among the remaining inner and outer hair cells (fig 9). Other histopathologic lesions included moderate glomerulonephritis. A moderate number of glomeruli were atrophic and the remaining glomeruli showed slightly thickened mesangium and capillary loops. Atrophy of the prostate, focal subacute prostatitis, and testicular degeneration were also present.

Monkey No. 451, treated with 25 consecutive im injections of neomycin sulfate, exhibited a transient weight loss of 9%, a transient moderate hemoglobin level of 10.2 g/100 ml, and a reversible moderate azotemia with a maximum BUN value of 30.9 mg/100 ml. The blood values returned to normal limits within 2 weeks after treatment. During treatment, the animal developed a hearing loss at pure tones of 18 kHz, which progressed to total irreversible deafness at high-frequency tones after Day 80. Hearing loss at 1 and 8 kHz appeared later than at high-frequency tones and progressed to a total irreversible deafness at 8 kHz and to an elevated hearing threshold at 1 kHz. The monkey was killed in good general condition 225 days after the start of treatment. Histopathologic lesions were found only in the inner ear; they were more severe than in the first monkey treated with neomycin sulfate (No. 397) and included a more severe loss of inner hair cells in the lower turns (table 1, fig 10).

The control monkey (No. 413) had no changes in hearing threshold throughout a testing period of 6 months, and surface preparations and a cochleogram showed minimal abnormalities (figs 2 and 3).

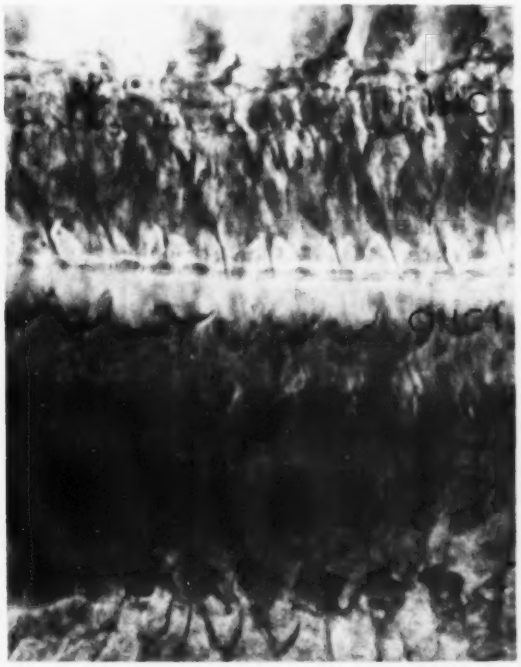

FIGURE 2.—Organ of Corti of untreated control monkey No. 413. Surface preparation of left ear, 1½ turns from base, phase-contrast photomicrograph. This monkey had no hearing loss during a 6-mo testing period. Note the intact inner hair cells (IHC) and the four rows of intact outer hair cells (OHC). Note the W-pattern of hairs which are in focus especially in row 1 of the OHC. Osmium tetroxide, × 1360.

DISCUSSION

Treatment with *cis*-dichlorodiammineplatinum (II) and neomycin sulfate leads to hearing loss in rhesus monkeys. *Cis*-dichlorodiammineplatinum (II) caused transient deafness in two of four monkeys but no detectable hearing loss in the other two animals. In one monkey, hearing loss appeared with azotemia during treatment and in the other it appeared after a latency of 3 months. In both monkeys normal hearing reoccurred within 200 days after treatment. In contrast, in both animals treated with neomycin sulfate delayed irreversible deafness occurred with a latency of approximately 3 months. One of the two animals had an early transient hearing loss which coincided with azotemia. The delayed onset of deafness and the observation that deafness for high-frequency tones preceded that for low-frequency tones agreed with earlier observations by Stebbins et al (18).

Both agents caused hair cell loss in all monkeys treated. In animals treated with *cis*-dichlorodiammineplatinum (II) the overall hair cell loss was by far not as severe as in monkeys treated with neomycin sulfate. Hair cell loss was most frequent among the outer hair cells in the lower turn and less frequent in the other turns. Inner hair cells were consistently less affected than the outer ones. In contrast, neomycin sulfate, in addition to a greater overall

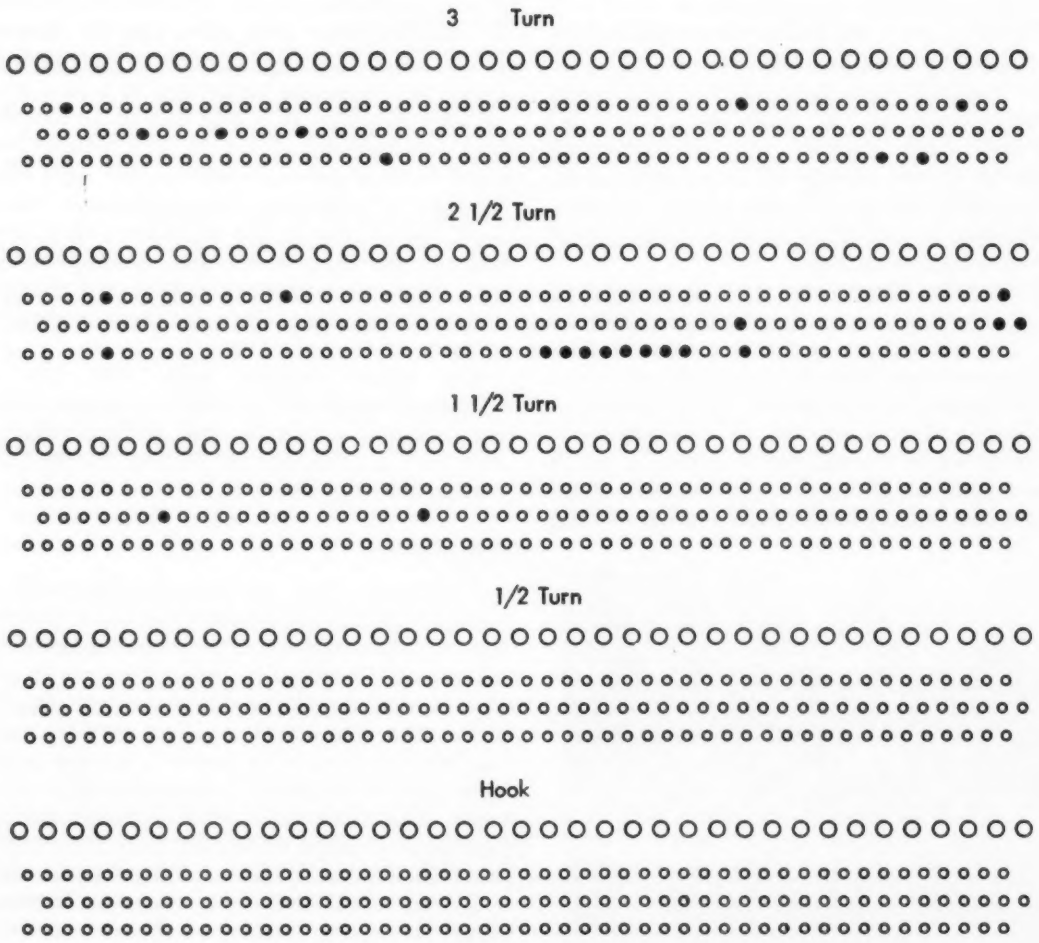

FIGURE 3.—Cochleogram: right organ of Corti of untreated control monkey No. 413. The diagram shows representative segments from hook to the 3rd turn. Circles represent top to bottom for each turn: inner hair cells (one row of large circles) and outer hair cells (three rows of small circles). Black circles are missing hair cells, empty circles are intact cells. Note very slight loss of outer hair cells in the upper turns.

hair cell loss, caused destruction primarily among the inner hair cells in the upper turn and, in one of the two animals treated, among the inner hair cells in the lower turn. Loss of outer hair cells was generally less severe and occurred primarily in the lower turn.

In addition to ototoxicity both agents caused other toxic manifestations. *Cis*-dichlorodiammineplatinum (II), in spite of less severe ototoxicity, caused a more severe overall toxicity than neomycin sulfate. Treatments with *cis*-dichlorodiammineplatinum (II) performed at the LD50 and at a dose which exceeded one half of the LD50 caused reversible hypocellularity of bone marrow and lymphoid tissues, and dysplasia of the renal tubular epithelium. In all monkeys the renal lesions developed into a persistent subacute glomerulonephritis and renal

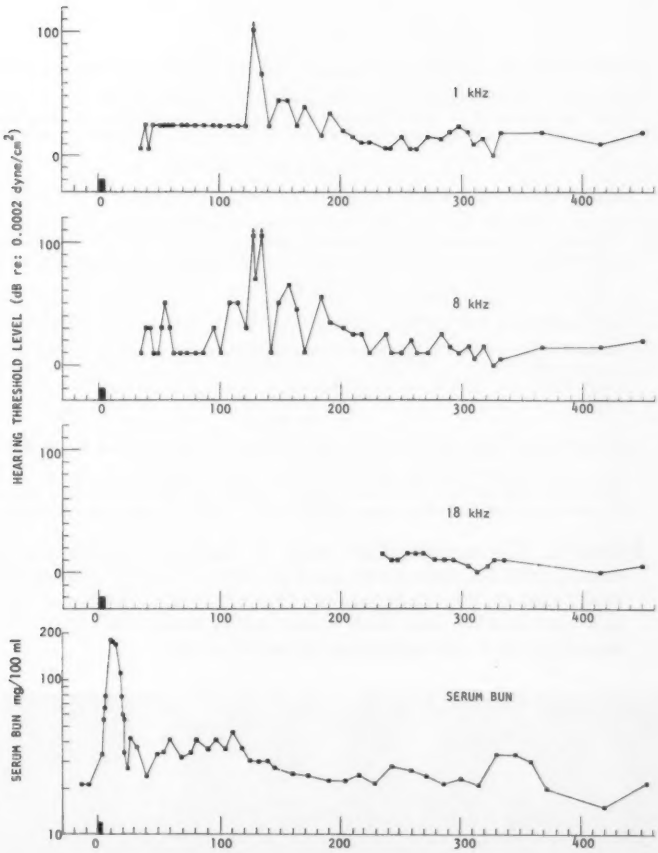

FIGURE 4.—Hearing threshold levels (right ear) for monkey No. 398 treated with *cis*-dichlorodiammineplatinum (II) (1.25 mg/kg/day iv for 5 days). Abscissa = treatment days. Bar indicates treatment period. Points with arrows indicate that the monkey was unable to hear at maximum sound pressure level produced by the audiometer. *Note*: The monkey had reversible hearing loss, severe loss of outer hair cells in the lower turns of the right cochlea, protracted azotemia, and glomerulonephritis.

atrophy. In contrast, neomycin sulfate did not lead to death in any animal. Renal toxicity also occurred and progressed to the same end-stage kidney lesions. It occurred, however, in only one of the two monkeys and was less severe than in animals treated with *cis*-dichlorodiammineplatinum (II).

Blood determinations at the peak of acute renal decompensation showed that treatment with both agents caused azotemia together with a decrease in the serum levels of K^+, Cl^-, and sometimes Ca^{++}. Differences were also noted: *cis*-dichlorodiammineplatinum (II), in the limited number of monkeys tested, produced a relative metabolic acidosis and hypocalcemia followed by hypercalcemia, whereas neomycin sulfate led to relative metabolic alkalosis and to hypocalcemia without secondary hypercalcemia.

The occurrence of more severe deafness after treatment with neomycin sulfate appeared to be related to destruction of a larger overall number of hair cells and to a greater ratio of loss of inner hair cells. Destruction of inner hair cells is particularly important for hearing,

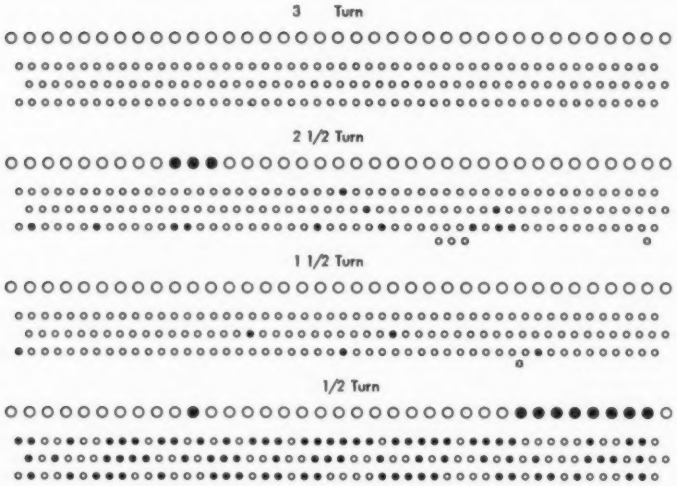

FIGURE 5.—Cochleogram: left organ of Corti of monkey No. 398 treated with five consecutive daily iv injections of *cis*-dichlorodiammineplatinum (II) (1.25 mg/kg). Note scattered pattern of inner and outer hair cell loss which is most severe in the lower turns. (*See* legend for fig 3 for explanation of cochleogram.)

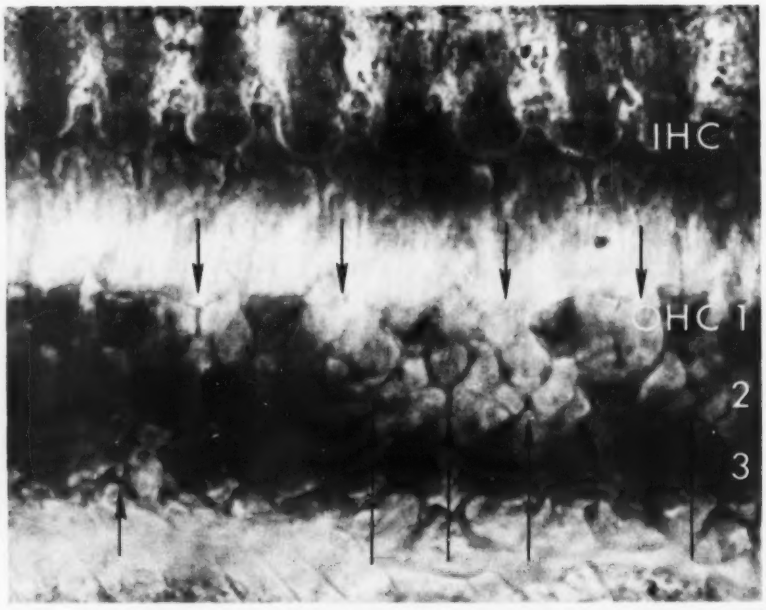

FIGURE 6.—Organ of Corti of monkey No. 398 treated with *cis*-dichlorodiammineplatinum (II). Surface preparation of right ear, ½ turn from the base, phase-contrast photomicrograph. This monkey received five daily consecutive iv injections of 1.25 mg/kg. Transient hearing loss developed 120–140 days after treatment and the animal was killed in apparently good general condition on Day 486. Note the intact inner hair cells (IHC) and the intact and missing (arrows) outer hair cells (OHC). Osmium tetroxide, × 1360.

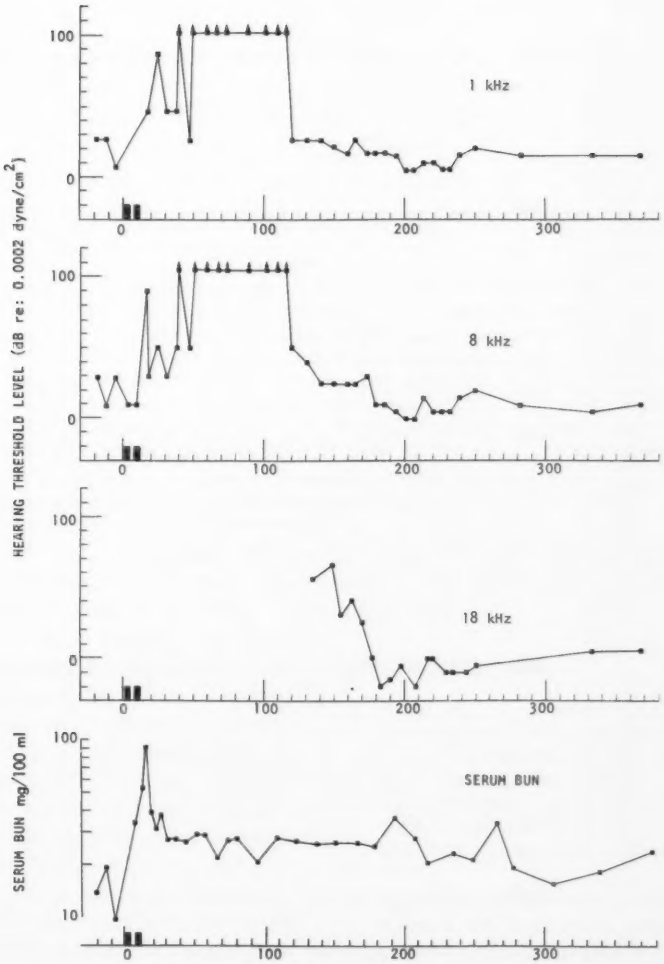

FIGURE 7.—Hearing threshold levels (right ear) for monkey No. 405 treated with *cis*-dichlorodiammineplatinum (II) (0.625 mg/kg iv **for 10 days). Abscissa = treatment days. Bar indicates treatment** period. Points with arrows indicate that the monkey was unable to **hear at maximum sound pressure level produced by the audiometer.** ***Note*: The monkey had reversible hearing loss, scattered loss of outer hair cells in the organ of Corti, irreversible azotemia, and renal histopathology.**

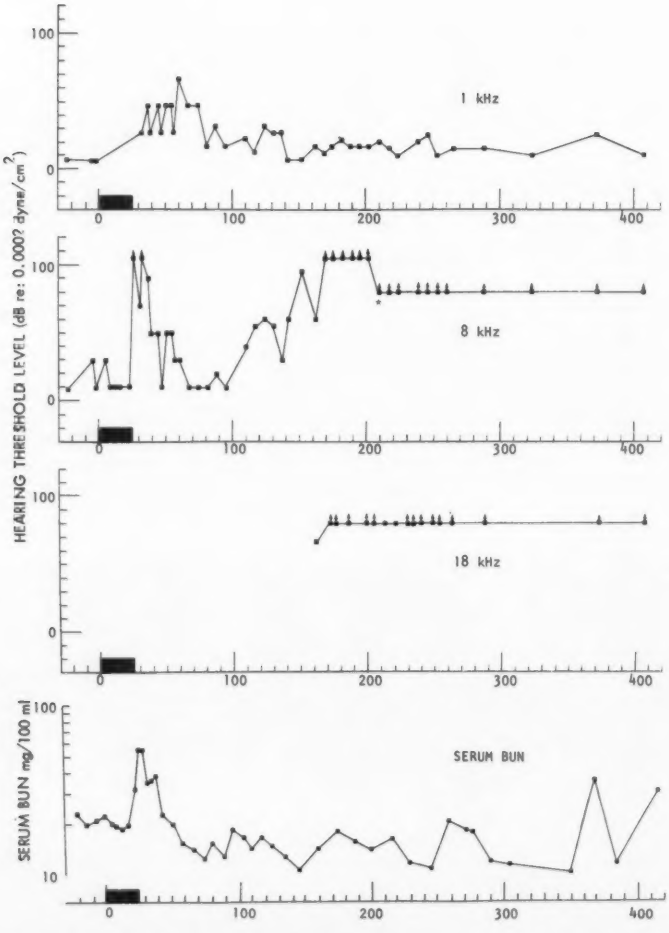

FIGURE 8.—Hearing threshold levels for monkey No. 397 treated with neomycin sulfate (50 mg/kg/day im for 25 days). Abscissa = treatment days. Bar indicates treatment period. Points with arrows indicate that the monkey was unable to hear at maximum sound pressure level produced by the audiometer. ★ = data obtained with a different audiometer. *Note*: The monkey had an early reversible phase of hearing loss coinciding with maximum BUN elevation. Irreversible hearing loss at 8 and 18 kHz began 4 mos after the start of treatment. The histopathologic evaluation revealed severe loss of inner and moderate loss of outer hair cells. Reversible azotemia and mild renal lesions were also noted.

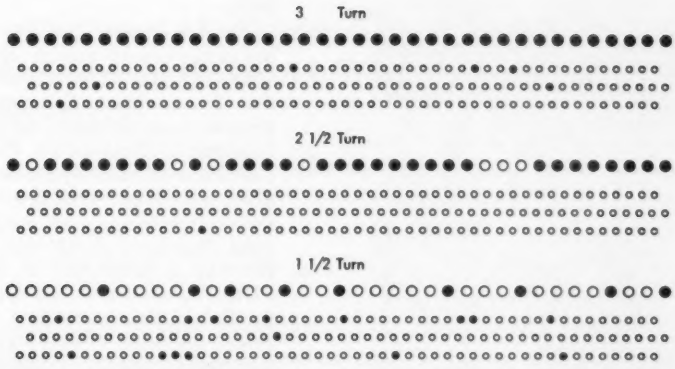

FIGURE 9.—Cochleogram: left organ of Corti of monkey No. 397 treated im with 25 daily consecutive doses of neomycin sulfate (50 mg/kg). This monkey was killed in fair general condition on Day 415. Histopathologic lesions included moderate glomerulonephritis and marked degeneration of testes and prostate. The monkey exhibited permanent deafness for pure tones of 8 and 18 kHz and reversible hearing loss for 1 kHz. Note scattered loss of outer hair cells and severe loss of inner hair cells in the upper turns of the right cochlea. The right cochleogram showed similar lesions. (*See* legend for fig 3 for explanation of cochleogram.)

because Spoendlin (19) has shown that 90% of the afferent fibers innervate inner hair cells while only 10% innervate outer hair cells.

The observations that rhesus monkeys treated with a dose as large as the LD50 developed only transient hearing loss whereas guinea pigs (20),[11] rats,[12] and man (6–9,21) became permanently deaf indicate that the monkey is less susceptible to ototoxicity than man and the other species. This conclusion agrees with earlier observations that kanamycin was more ototoxic in man (22) than in monkeys (18).

The present study demonstrates differences in the effects of *cis*-dichlorodiammineplatinum (II) and neomycin sulfate which include differences in the characteristics of hearing loss and in the distribution of the lesions in the organ of Corti. Possible differences in the pathogenesis of the acute phase of renal toxicity were also noted.

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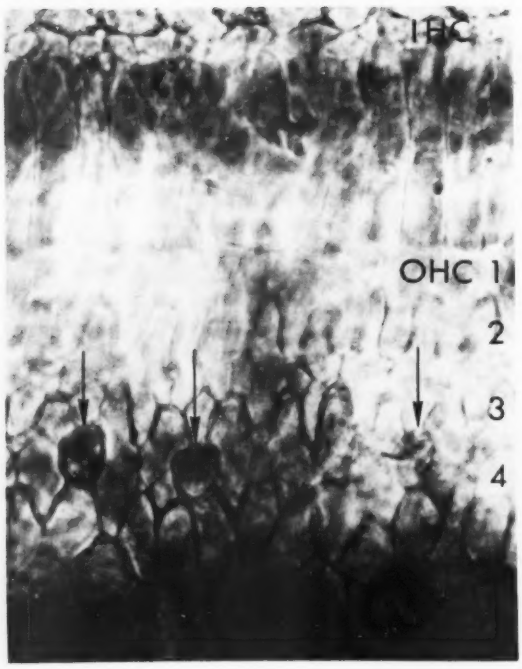

FIGURE 10.—Organ of Corti of monkey No. 451 treated with neomycin sulfate. Surface preparation of right ear, 1½ turns from the base, phase-contrast photomicrograph. This monkey received 25 daily consecutive im injections of 50 mg/kg. Total irreversible deafness for high-frequency tones developed after Day 80 of treatment and the animal was killed in good general condition on Day 225. Note the destruction of all inner hair cells (IHC) with replacement by reticular scars. Only three outer hair cells (OHC) remain (arrows) in the otherwise empty reticular framework. Osmium tetroxide, × 1360.

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L-[αS, 5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic Acid (NSC-163501): A New Amino Acid Antibiotic With the Properties of an Antagonist of L-Glutamine [1,2,3]

Hiremagalur N. Jayaram,[4] David A. Cooney,[4] Joan A. Ryan,[5] Gary Neil,[6] Robert L. Dion,[4] and Vincent H. Bono [4,7,8]

SUMMARY

L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501), an antibiotic elaborated by *Streptomyces sviceus*, has been shown to be a powerful inhibitor of many mammalian and bacterial reactions involving the transfer of nitrogen from the γ-carboxamide of L-glutamine. Thus, the utilization of L-glutamine for the synthesis of carbamyl phosphate, L-asparagine, guanosine-5′-monophosphate, cytidine-5′-triphosphate, *N*-formylglycinamidine ribonucleotide, NAD, glucosamine-6-phosphate, and anthranilic acid is strongly or totally inhibited by a concentration of NSC-163501 of 1×10^{-3} M. L-Glutamate synthetase of *Escherichia coli* is only modestly inhibited and 5-phosphoribosylamine synthesis in fetal rat liver is comparatively refractory to inhibition. NSC-163501 treatment of L1210 cells growing in a low L-glutamine culture medium produced arrest in G_1 or early S phase. Of the amino acids tested, only L-glutamine antagonized such growth inhibition.

[Cancer Chemother Rep 59:481–491, 1975]

L-Glutamine occupies an important role in the biosynthesis of a comparatively large number of indispensible metabolites (1). Similarly, analogs of L-glutamine with the ability to inhibit these biosynthetic reactions have assumed great importance as biochemical tools. 6-Diazo-5-oxo-L-norleucine (DON), azaserine, albizzin, and γ-glutamyl hydrazide are prominent on a list of such inhibitory analogs (1,2). Used in vivo, these agents dramatically reveal the biochemical, toxicologic, and oncologic consequences of the interruption of L-glutamine utilization. By the same token, each of these agents has a different spectrum of inhibitory activity against the several enzymes under discussion. For example, DON is a powerful inhibitor of the reaction wherein L-glutamine transfers its

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[2] Supported in part by contracts NO1-CM-33728 and NO1-CM-33707 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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[7] The authors wish to thank Miss S. L. Kuentzel for her excellent technical assistance, and Mrs. Ruth Davis, Mrs. Linda Klipp, and Mrs. Audrey Loveridge for their assistance in the preparation of the manuscript.

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amide to L-aspartic acid (L-asparagine synthetase), whereas azaserine is inert with respect to this reaction (2). It is the purpose of the present communication to report that NSC-163501 and its hydroxy analog, NSC-176324, compounds previously reported to be powerful inhibitors of the L-asparagine synthetase of murine tumor and pancreas (3), can also inhibit several other enzymes catalyzing amidotransfer from L-glutamine.

MATERIALS AND METHODS

L-[U-^{14}C]glutamine (specific activity, 50 microcuries/μmol) was purchased from Amersham/Searle Corp., Arlington Heights, Illinois. *N*-formylglycinamide ribonucleotide was a generous gift of Dr. John Buchanan, Massachusetts Institute of Technology, Cambridge, and desamido-NAD was donated by Dr. Alan Berg, Arthur D. Little, Inc., Cambridge, Massachusetts. Xanthosine-5′-monophosphate (XMP), uridine-5′-triphosphate (UTP), 5-phosphoribosyl pyrophosphate, and D-fructose-6-phosphate were all purchased from Sigma Chemical Co., St. Louis, Missouri.

Sources of the Bacterial Enzymes

L-Glutamate synthetase (specific activity, 26.2 IU/mg of protein) and L-glutamine synthetase (specific activity, 10 IU/mg of protein), both from *Escherichia coli*, were kindly supplied as gifts by Dr. Earl Stadtman, National Institutes of Health, Bethesda, Maryland; anthranilate synthetase from *Serratia marcescens* (specific activity, 0.462 IU/mg of protein) was donated by Dr. Howard Zalkin of Purdue University, Lafayette, Indiana; and carbamyl phosphate synthetase from *E. coli* (specific activity, 50 IU/mg of protein) was generously provided by Drs. Vaira Wellner and Alton Meister of Cornell University School of Medicine, New York, New York. L-Glutaminase from *E. coli* (specific activity, 0.6 IU/mg of protein) was purchased from Worthington Biochemical Corp., Freehold, New Jersey. For storage, the enzyme was taken up in 50% glycerine; the resulting suspension was rendered 0.001 M in sodium acetate (pH 5.0) and was stored at 0°C. For assay, 5 μl of a 1:10 dilution of the stock L-glutaminase was used. L-Asparaginase from *E. coli* (specific activity: 340 IU/mg of protein with L-asparagine as substrate, 10 IU/mg of protein with L-glutamine as substrate) was generously supplied as a gift by Merck Sharp and Dohme, West Point, Pennsylvania.

L-Asparagine synthetase was partially purified from a variant of L5178Y leukemia resistant to L-asparaginase (L5178Y/AR) as described in a previous communication (3). All of the other mammalian enzymes were examined in fetal rat liver. For this purpose, livers from 20 midterm fetuses were homogenized in 0.1 M tris-HCl buffer (pH 7.4) containing 0.5 mM EDTA and 0.1 mM dithiothreitol. After centrifugation at 105,000 *g* for 30 minutes the supernatant was dialyzed for 6 hours against three changes of 1 liter each of homogenization buffer. This step is necessary to remove endogenous substrates.

Assay of Amidotransfer From L-Glutamine

This assay measures the substrate-dependent (acceptor-dependent) breakdown of L-[U-^{14}C]-glutamine to L-[U-^{14}C]glutamic acid. The following were admixed in a final volume of 25 μl: 1 microcurie of L-[U-^{14}C]glutamine (final concentration, 0.2 mM), neutralized acceptor (to achieve a final concentration of 0.01 M), neutralized ATP and $MgCl_2$ (each at 0.02 M), and 10 μl of enzyme (to initiate the reaction). Two sets of blanks were constituted: the first received all ingredients except the specified acceptor (amide-recipient), while the second received all ingredients except ATP and $MgCl_2$. The first of these blanks allowed an estimation of the basal L-glutaminase activity of the extract, and the second allowed an estimation of ATP-independent amidotransfer (ATP · $MgCl_2$ is not required for the synthesis of D-fructose-6-phosphate, 5-phosphoribosylamine, or L-glutamate). After 30 minutes at 37°C the reaction mixtures were heated at 95°C for 5 minutes and cooled. Any L-[U-^{14}C]glutamic acid produced in the incubation step was then quantitated by the addition of 50 μl of 0.66 M acetate buffer (pH 4.2) containing 1 IU of L-glutamate decarboxylase purified from *E. coli* by the method of Shukuya and Schwert (4). The [^{14}C]O_2 resulting from this step was collected in 5-μl droplets

of 40% NaOH deposited on the underside of the lid of the reaction vessel, essentially as described by Cooney et al (5).

In the calculations of activities, the basal rate of hydrolysis of L-glutamine was subtracted from the rate seen in the presence of acceptor.

None of the inhibitors tested interfered with the reagent enzymes, L-glutamate decarboxylase or L-asparaginase, under the conditions of this assay. It should be pointed out, too, that in systems such as these, "% inhibition" has no precise definition in kinetic terms. Competitive inhibition, irreversible inhibition, time-dependent inhibition, and substrate-promoted inhibition may all be occurring. In addition, no attempt has been made to verify either the degree of saturation of the several enzymes studied with the concentration of L-glutamine used or the linearity of the rate measurements with respect to time and enzyme.

L-Glutamine synthetase was measured radiometrically. The reaction mixture allows the synthesis of L-[1-^{14}C]glutamine from L-[1-^{14}C]-glutamic acid in the presence of a mixture of $MgCl_2$, NH_4Cl, and ATP. Five μl of a 1:10 (wt/vol) tissue homogenate or bacterial extract was incubated at 37°C for 10 minutes with 5 μl of a substrate mixture containing 0.02 M ATP, 0.02 M $MgCl_2$, 0.04 M L-glutamic acid, 0.25 microcuries of L-[1-^{14}C]glutamic acid, and 0.16 M NH_4Cl. The reaction was terminated by heating at 95°C for 3 minutes, and the reaction vessels were then centrifuged for 3 minutes at 12,000 *g* to separate any denatured protein. Unreacted L-glutamic acid was removed by incubation in open vessels with 1 IU of L-glutamate decarboxylase in 50 μl of 0.33 M acetate buffer (pH 4.2) for 2 hours at 37°C. Thereafter, L-[1-^{14}C]-glutamine was hydrolyzed to L-[1-^{14}C]glutamate by the addition of 20 IU of L-asparaginase to each vessel. The [^{14}C]O_2 was collected in 5-μl droplets of 40% KOH placed on the underside of the lid (5). Appropriate reagent blanks and standards were included in each experiment. After 3 hours at 37°C, the lids were removed and counted in an ethanol-toluene (1:2, vol/vol) scintillant containing Liquifluor from New England Nuclear Corp., Boston, Massachusetts.

Tissue Culture Methodology

For the studies on the effects of amino acids on the inhibitory activity of NSC-163501 towards L1210 cell growth in culture, RPMI 1634 medium (6), lacking one of each of the following amino acids, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, glutathione (reduced), L-histidine, L-leucine, L-methionine, L-phenylalanine, or L-proline, was prepared. The medium was completed in the usual manner with penicillin (0.1 mg/ml), streptomycin (0.05 mg/ml), $NaHCO_3$ (0.075% wt/vol), and fetal calf serum (5%). The desired amino acid was then added at the required concentration. Adjustment of pH was made with NaOH. L1210 cells (about 5×10^5 cells/ml in complete medium) were then added to the test medium to yield a concentration of 5×10^3 cells/ml. This cell suspension was added to test tubes containing saline (control) or solutions of NSC-163501 in saline. Growth of cells (70 hours at 37°C) was determined by electronic particle counting using the method of Buskirk (7). For each amino acid concentration, growth in the presence of NSC-163501 was compared to that in the control cultures according to the formula:

$$\% \text{ of control} = \frac{Cs - Co}{Cc - Co} \times 100$$

where Co = cell concentration at zero time (about 5×10^3 cells/ml), Cs = cell concentration of samples containing NSC-163501 after 70 hours at 37°C, and Cc = cell concentration of control samples after 70 hours at 37°C. Tests to ascertain the effects of varied concentrations of other amino acids on the activity of NSC-163501 were then carried out according to the scheme shown in table 1.

Measurement of the DNA Content of L1210 Cells in Culture

L1210 cells growing in RPMI 1630 medium supplemented with 20% fetal calf serum, penicillin, and streptomycin were transferred to a modified RPMI 1630 medium in which the concentration of L-glutamine was reduced to 60 μg/ml (25% of the usual concentration) and treated with NSC-163501 at doses of either 0.113 or 1.13 μg/ml. Under these conditions there was no significant change in doubling time over a 24-hour period for an untreated control as compared to a control growing in me-

TABLE 1.—Concentrations of amino acids used in studies of the cytocidal activity of NSC-163501

Amino acid	Conventional concentration in RPMI 1634 medium (μg/ml)	Concentration ranges for "normal" L1210 cell growth* (μg/ml)	Concentration ranges studied for effect on NSC-163501 inhibition (μg/ml)
L-Arginine	100	100–3000	100–10,000
L-Asparagine	30	0–300†	0–300†
L-Aspartic acid	30	0–300†	0–300†
L-Cysteine	100	100–3000	100–10,000
L-Glutamic acid	80	0–200†	0–800†
L-Glutamine	300	100–1000	0–3000†
Glutathione (reduced)	10	3–100	0–300†
Glycine	15	15–450	15–1500
L-Histidine	50	1–500	0–500†
L-Isoleucine	50	50–5000	50–5000
L-Lysine-HCl	75	75–2250	75–7500
L-Methionine	15	5–150	0–150†
L-Phenylalanine	30	3–100	0–300†
L-Proline	30	0–300†	0–300†
L-Serine	50	50–500	50–5000
L-Threonine	50	50–500	50–5000
L-Tryptophan	10	10–1000	10–1000
L-Tyrosine	30	30–3000	30–3000
L-Valine	40	40–4000	40–4000

*Less than 10% inhibition or stimulation of growth of L1210 cells compared with cell growth at conventional concentration.

†The "0" level indicates that no additional amino acid was added. The fetal calf serum in the medium certainly contains amino acids; the cell inoculum would contribute less than 1% of the normal concentration of amino acids or metabolites.

dium containing 240 μg of L-glutamine/ml. Cell viabilities were assessed by trypan-blue exclusion.

After 6- and 24-hour periods of drug exposure, aliquots of untreated and drug-treated cultures were fixed and stained with benzoflavine for flow microfluorometry using a procedure modified from that employed by Tobey et al (8). Modifications consisted of: (*a*) elimination of the trypsin/deoxyribonuclease digestion prior to fixation; (*b*) addition of 0.5% Triton-X-100 to all solutions utilized in the staining procedures to enhance monodispersion; (*c*) use of 0.02% benzoflavine in lieu of 0.03% acriflavine; and (*d*) use of an equal number of cells for each sample during the staining process. The last modification requires explanation. In extensive previous experiments, we have shown that there is a direct relationship between the number of cells used during the staining process and the final intensity of their fluoresence emission. Details of these phenomena are to be published elsewhere.

Stained cells were analyzed in a Biophysics Cytofluorograf 4801 (Bio/Physics Systems, Inc., Mahopac, NY) coupled with a Northern Scientific Pulse Height Analyzer 633 (Northern Scientific, Inc., Middleton, Wis); 100,000 cells were analyzed for each sample.

RESULTS

Table 2 summarizes the inhibitory potency of NSC-163501 and its hydroxy analog, NSC-176324, versus a panel of L-glutamine-utilizing amidotransferases present in dialyzed extracts of midterm fetal rat livers. It can be appreciated that both antibiotics interfere with the synthesis of carbamyl phosphate, L-asparagine, NAD, guanosine-5′-monophosphate, cytidine-5′-triphosphate, glucosamine-6-phosphate, and *N*-formylglycinamidine ribonucleotide, but that ribosylamine-5-phosphate:pyrophosphate phos-

phoribosyltransferase (5-phosphoribosylamine synthetase) is comparatively resistant to inhibition. It is also apparent that the ring-hydroxylated isoxazole, NSC-176324, is generally a less powerful inhibitor than NSC-163501. In this context, it is worthwhile pointing out that NSC-176324 appears to be a somewhat less potent antitumor agent than the parent drug (9).

Because certain amidotransfer reactions are absent from mammalian systems, a select number of bacterial amidotransferases also were examined. Both isoxazoles inhibited L-glutamine-dependent carbamyl phosphate synthetase of *E. coli* very powerfully, but L-glutamate synthetase from the same source was only modestly inhibited. In separate studies it has been found that preincubation of this enzyme with inhi-

TABLE 2.—Inhibition of several amidotransferase enzymes by NSC-163501 and NSC-176324*

E.C. No.	Reactions	Inhibition (%) by— NSC-163501	NSC-176324
	Mammalian enzymes		
2.7.2.9	ATP: carbamate phosphotransferase	70	50
6.3.5.4	L-Aspartate: L-glutamine amido-ligase	76	65
6.3.5.1	Desamido-NAD: L-glutamine amido-ligase	100	100
6.3.5.2	XMP: L-glutamine amido-ligase	92	75
6.3.4.2	UTP: L-glutamine amido-ligase	100	64
5.3.1.19	L-Glutamine: D-fructose-6-phosphate aminotransferase	76	60
2.4.2.14	Ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase	11	21
6.3.5.3	*N*-formylglycinamide ribonucleotide: L-glutamine amido-ligase	86	71
	Bacterial enzymes		
2.6.1.53	L-Glutamate synthetase	60	58
2.7.2.9	Carbamyl phosphate synthetase: L-glutaminase activity	98	100
2.7.2.9	Carbamyl phosphate synthetase: carbamyl phosphate synthesis	98	100
4.1.3.27	Anthranilate synthetase: L-glutaminase activity	98	100
4.1.3.27	Anthranilate synthetase: anthranilate synthesis	98	100

*The substrate-dependent hydrolysis of L-glutamine was carried out as described in the Materials and Methods section. Inhibitors were present at a final concentration of 1×10^{-3} M. In the case of L-glutamate synthetase, NADPH and 2-oxoglutarate were used at a concentration of 0.01 M each. The rates of the uninhibited reactions in the case of mammalian enzymes were 607, 510, 729, 1757, 484, 518, 1177, and 1969 picomols of L-glutamine utilized/mg of protein/hr in the presence of sodium bicarbonate, L-aspartate, desamido-NAD, XMP, UTP, D-fructose-6-phosphate, 5-phosphoribosyl pyrophosphate, and *N*-formylglycinamide ribonucleotide respectively. The specific activities of the bacterial enzymes were essentially the same as given in the Materials and Methods section.

bitor, in the presence or absence of L-glutamine (0.01 M), significantly augments the degree of inhibition seen.

Although NSC-176324 proved to be a potent inhibitor of the bacterial amidotransferases examined in the present study, this ring-hydroxylated isoxazole, in contradistinction to NSC-163501, is virtually devoid of antibacterial activity (9). This finding warrants the conclusion that the antibacterial activity of NSC-163501 is not mediated by its inhibition of the synthesis of carbamyl phosphate, anthranilic acid, or L-glutamate.

In table 3, the inhibitory activity of NSC-163501 has been compared with that of several prototypical antagonists of L-glutamine: azaserine, DON, and 5-chloro-4-oxo-L-norvaline (CONV). Each inhibitor was examined at a final concentration of 1×10^{-3} M; fetal rat liver served as the source of most of the enzymes examined. DON and CONV inhibited all of the reactions studied and proved, therefore, to be the most universal antagonists of the group. The isoxazole, NSC-163501, again was found to inhibit all of the amidotransferases except 5-phosphoribosylamine synthetase. Azaserine proved to be the most selective of the agents examined, producing strong inhibition of the synthesis of *N*-formylglycinamidine ribonucleotide, significant inhibition of the synthesis of glucosamine-6-phosphate and CTP, but feeble inhibition of the synthesis of NAD and the hydrolysis of L-glutamine. The remaining reactions studied were, however, totally resistant to inhibition by azaserine.

That the inhibition exerted by NSC-163501 did not extend to all reactions utilizing or producing L-glutamine is shown by the data presented in table 4. Thus, L-glutamine synthetase from such diverse sources as rat liver, mouse

TABLE 3.—Comparison of several L-glutamine antagonists as inhibitors of select amidotransfer reactions catalyzed by enzymes in fetal rat liver or *Escherichia coli**

E.C. No.	Enzyme	Inhibition (%) by—			
		NSC-163501	DON	CONV	Azaserine
3.5.1.2	L-Glutamine amido-hydrolase	58	80	86	19
6.3.5.4	L-Aspartate: L-glutamine amido-ligase	95	100	86	0
6.3.5.1	Desamido-NAD: L-glutamine amido-ligase	90	74	73	28
6.3.5.3	*N*-formylglycinamide ribonucleotide: L-glutamine amido-ligase	99	100	100	98
6.3.5.2	XMP: L-glutamine amido-ligase	99	100	94	0
5.3.1.19	L-Glutamine: D-fructose-6-phosphate aminotransferase	96	93	96	61
2.4.2.14	Ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase	10	100	99	3
2.7.2.9	ATP: carbamate phosphotransferase†	99	55	61	0
6.3.4.2	UTP: L-glutamine amido-ligase	96	82	99	57

*Inhibitors were added to a final concentration of 1×10^{-3} M. The substrate-dependent hydrolysis of L-glutamine was carried out as described in the Materials and Methods section. The uninhibited rates were substantially the same as those listed in the footnote to table 2.

†The enzyme from *E. coli* was used for this study.

liver, and *E. coli* proved to be refractory to inhibition by NSC-163501. On the other hand, although they are not amidotransferases, the L-glutaminases and L-glutamine-α-keto acid transaminase of fetal rat liver were powerfully inhibited by this isoxazole. Some comment on the inhibition of the so-called crude neutral L-glutaminase of *E. coli* is in order (10). Inasmuch as several L-glutamine amidotransferases can function as simple L-glutaminases, even in the absence of other substrates, the activity examined here may, in fact, reflect amidohydrolysis by this group of enzymes and may not be due to L-glutaminase active at pH 7.4. When the inhibitory action of NSC-163501 was examined versus the prototypical L-glutaminase of *E. coli*, whose pH optimum falls at 5.0 and which is virtually inert at pH 7.4, only marginal inhibition was observed at a concentration of 1×10^{-3} M. On the other hand, the crude bacterial "L-glutaminase" active at neutral pH was inhibited significantly under these conditions.

The observations presented in table 5 are congruent with the conclusion that NSC-163501 impairs the utilization of L-glutamine in vivo to a far greater degree than its synthesis. For these studies, BDF_1 mice bearing subcutaneous nodules of L5178Y/AR leukemia were given intraperitoneal (ip) injections of NSC-163501 (100 mg/kg) and were killed 4 hours later. Enzymatic analysis of the concentration of L-glutamine in the pancreas of the mice revealed a

TABLE 4.—In vitro inhibition by NSC-163501 of enzymes hydrolyzing and synthesizing L-glutamine

Enzyme*	Source	Inhibition (%)
L-Glutaminase, phosphate-independent	Fetal rat liver	76
L-Glutaminase, phosphate-independent plus maleate	Fetal rat liver	82
L-Glutaminase, phosphate-dependent	Fetal rat liver	81
"L-Glutaminase" (pH 7.4)	*E. coli*	60
L-Glutaminase (pH 5.0)	*E. coli*	6
L-Glutamine-α-keto acid transaminase	Fetal rat liver	52
L-Glutamine synthetase	Fetal rat liver	0
L-Glutamine synthetase	Adult rat liver	0
L-Glutamine synthetase	*E. coli*	0

**Mammalian enzymes:* Rat livers were homogenized in 0.1 M tris-HCl buffer (pH 7.4) containing 0.1 mM dithiothreitol and 0.5 mM EDTA, and were then centrifuged at 105,000 *g* for 30 mins. The supernatant was dialyzed against three changes of 1 liter each of the homogenization buffer at 4°C, and used as such for the assays indicated. To measure phosphate-independent L-glutaminase, 10 μl of 0.1 M tris-HCl buffer (pH 8.0) was added to 5 μl (0.25 microcuries) of L-[U-^{14}C]glutamine. The mixture was incubated at 37°C for 30 mins and the production of L-[U-^{14}C]glutamate was quantitated by the addition of L-glutamate decarboxylase in 0.2 M acetate buffer (pH 4.2). [^{14}C]O_2 was trapped as described earlier (5). To measure maleate stimulation of phosphate-independent L-glutaminase, maleate was added to the above system to a final molarity of 0.02 M. To measure phosphate-dependent L-glutaminase, sodium phosphate was added to the above system to a final molarity of 0.01 M. L-Glutamine-α-keto acid transaminase was measured radiometrically. In a final volume of 25 μl were admixed: liver supernatant (10 μl); 0.05 M sodium phosphate (5 μl); L-[U-^{14}C]glutamine (5 μl, 0.25 microcuries); tris-HCl buffer (0.1 M, pH 8.0, 5 μl); and inhibitor or water. After 30 mins at 37°C, the keto acids produced (a mixture of α-ketoglutaramate and α-ketoglutarate) were α-decarboxylated by the addition of 50 μl of 1 N HCl containing 1% H_2O_2. [^{14}C]O_2 was trapped as described earlier (5). L-Glutamine synthetase was measured as described in the Materials and Methods section.

Bacterial enzymes: The "L-glutaminase" (pH 7.4) was a 105,000 g supernatant of a crude extract of *E. coli* prepared by disruption of the cells at 4°C in 0.1 M tris-HCl buffer (pH 7.4). The sources of the remaining bacterial enzymes are given in the Materials and Methods section.

Inhibitor: NSC-163501 was added to a final concentration of 1 mM in all cases.

400% elevation over the levels measured in saline controls. Inasmuch as the rodent pancreas is known to import and utilize L-glutamine at a rate greater than virtually any other organ (11), this finding is taken as evidence for the pharmacologic interruption of one or both of these processes. It is noteworthy that the drug failed to elevate the levels of L-glutamine in liver.

Pursuant to the observations that the isoxazole antibiotics profoundly deranged the metabolism of L-glutamine in the pancreas of the mouse, automatic amino acid analysis of pancreatic extracts was carried out to determine whether the metabolism of certain other amino acids was also disturbed. The results of these studies, presented in table 6, demonstrate that L-threonine increases to an even greater degree than L-glutamine in treated mice. The concentrations of L-aspartic acid and L-glutamic acid, on the other hand, were little affected by the drug.

When the effects of various amino acids on the antibacterial activity of NSC-163501 were

TABLE 5.—Influence of a single parenteral dose of NSC-163501 on the concentration of L-glutamine in the pancreas, brain, liver, and tumor of mice bearing L5178Y/AR leukemia*

	Concentration of L-glutamine (nmol/g wet weight)	
Organ	NSC-163501	Saline
Pancreas	3263 ± 99	970 ± 80
Brain	4035 ± 301	3293 ± 200
Liver	2454 ± 108	2660 ± 124
Tumor	613 ± 23	615 ± 21

*Groups of six BDF_1 mice bearing subcutaneous nodules of L5178Y/AR leukemia (resistant to L-asparaginase) were given a single ip injection of NSC-163501 at a dose of 100 mg/kg. Four hours later the pancreas, brain, liver, and tumor were removed and homogenized 1:3 (wt/vol) in 0.1 M tris-HCl buffer (pH 7.4) containing 0.5 mM EDTA. Analysis of the L-glutamine concentrations was carried out enzymatically (11). Tabulated are the means ± standard deviations.

TABLE 6.—Influence of a single parenteral dose of NSC-163501 on the concentration of select amino acids in the pancreas of the mouse*

	Concentration (nmol/g wet weight)		Ratio of
Amino acid	NSC-163501	Saline	NSC-163501:saline
L-Aspartic acid	940 ± 20	810 ± 200	1.16
L-Threonine	1230 ± 85	200 ± 30	6.15
L-Serine	1000 ± 10	595 ± 18	1.68
L-Asparagine	91 ± 5	132 ± 16	0.69
L-Glutamic acid	2410 ± 120	2645 ± 305	0.90
L-Glutamine	3435 ± 95	1210 ± 200	2.83
Glycine	4730 ± 20	3520 ± 180	1.343
L-Alanine	2275 ± 55	1590 ± 90	1.43

*Groups of six BDF_1 mice were given a single ip injection of NSC-163501 at a dose of 100 mg/kg. Four hours later their pancreata were removed and homogenized in 3 volumes (wt/vol) of 0.1 M tris-HCl buffer (pH 7.4) containing 0.5 mM EDTA. The homogenates were heated at 95°C for 10 minutes, centrifuged at 12,000 *g* for 9 minutes, and the supernatants diluted 1:25 (vol/vol) with deionized water. Amino acid analysis was carried out on the Jeolco amino acid analyzer using the lithium citrate system of Benson et al (13). Tabulated are the means ± standard deviations.

examined, it was observed that these effects were antagonized most strongly by L-histidine and to a lesser degree by deoxyinosine (14). Inasmuch as NSC-163501 and NSC-176324 also inhibit the growth of L1210 cells in culture (9,14), we have investigated the effects of amino acids on the L1210 growth-inhibitory activity of NSC-163501. With one exception, even at concentrations at least tenfold higher than those normally present in the medium (RPMI 1634), no amino acids, including L-histidine, significantly alter the growth-inhibitory effect of NSC-163501. However, L-glutamine did exert an effect (table 7). The concentration-dependent inhibitory effect of NSC-163501 on L1210 cell growth was more pronounced in the presence of 100 μg/ml of L-glutamine than at the conventional medium concentration of 300 μg/ml. At a higher-than-normal level of L-glutamine (1000 μg/ml) the growth-inhibitory activity of NSC-163501 was reduced. It is noteworthy that control cell growth was approximately equal over the tenfold L-glutamine concentration range used. Significant reduction in *control* cell growth, observed when L-glutamine concentrations higher or lower than those shown in table 7 were employed (eg, 3000 μg/ml), precluded rigorous comparison of results obtained at those L-glutamine concentrations. However, the data obtained were consistent with the antagonistic effect noted above.

A number of other amino acids, not present in RPMI 1634 medium, were also tested for their ability to influence inhibition of L1210 cells by NSC-163501. β-Alanine (1000 μg/ml), L-hydroxyproline (100 μg/ml), L-cysteine (10 μg/ml), and L-2-pyrrolidone-5-carboxylic acid (100 μg/ml) all failed to alter the inhibition exerted by NSC-163501.

The effect of 6 and 24 hours of exposure to NSC-163501 on cell-cycle progression, as measured by the DNA content of L1210 cells in culture, is shown in figure 1. The first major peak in this figure, labeled 2C, represents cells with a G_1 DNA content; a second peak at twice the distance from the origin, labeled 4C, represents cells with a G_2 + M DNA content. Cells in S phase, containing intermediate amounts of DNA, are distributed between these two peaks.

At the lower drug concentration (0.113 μg/ml), little effect was seen at 6 hours. Even after 24 hours of exposure, viability remained at 97% of control but there was a decrease in the percentage of cells in G_2 with a concomitant increase of those in G_1 or early S phase. At this drug concentration the growth rate was 50% of control.

At the higher drug concentration (1.13 μg/ml) the growth rate was 20% of control. The percent of cells in G_2 was decreased and the percent of cells in G_1 or early S was increased after only 6 hours of exposure. By 24 hours, viability had dropped to 87% of control and degradation of DNA had occurred as evidenced by the presence of a pre-G_1 population. This population appears to originate from cells arrested by the antibiotic in G_1 or early S phase.

It should be noted, too, that the control cells

TABLE 7.—Effect of L-glutamine on the inhibition by NSC-163501 of growth of L1210 cells in culture*

	L-Glutamine (μg/ml)					
	100		300		1000	
NSC-163501 (μg/ml)	70-Hr cell concentration (10^3 cells/ml)	Inhibition of growth (%)	70-Hr cell concentration (10^3 cells/ml)	Inhibition of growth (%)	70-Hr cell concentration (10^3 cells/ml)	Inhibition of growth (%)
0	429	—	453	—	462	—
0.0625	100	78	298	35	472	0
0.125	39.3	92	128	73	254	46
0.25	10.5	98.8	38.5	92.6	74.4	85
0.50	7.8	99.5	13.5	98.3	17.4	97.4

*L1210 cells (5.6×10^3/ml) in complete RPMI 1634 media containing various concentrations of L-glutamine (100, 300, or 1000 μg/ml) were incubated with NSC-163501 or saline (controls). After 70 hrs' incubation at 37°C, the concentration of cells was determined by electronic particle counting and the percentage of control growth was calculated as described in the Materials and Methods section. The conventional L-glutamine concentration employed in RPMI 1634 medium is 300 μg/ml.

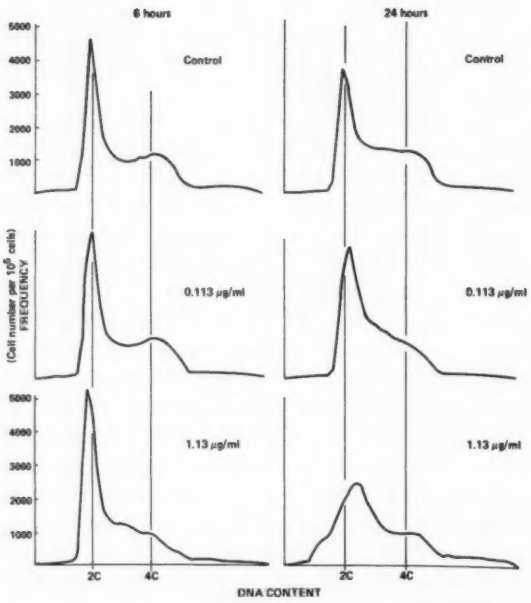

FIGURE 1.—DNA distribution patterns showing frequency of individual cellular DNA content in L1210 cells grown in "reduced L-glutamine" RPMI 1630 media (60 μg/ml) and treated with NSC-163501 at the concentrations and for the periods indicated in the Materials and Methods section. Ordinate: frequency of a given DNA content expressed in cell number per 10^6 cells. Abscissa: relative fluorescence emission (proportional to DNA content).

grown in the presence of low levels of L-glutamine (60 μg/ml) show a shift in cell distribution into the S phase from the 6-hour to the 24-hour samples. This effect, not seen in cells growing in RPMI 1630 media containing L-glutamine at a concentration of 240 μg/ml, is presently being investigated.

DISCUSSION

In the present study, the new oncolytic antibiotic, NSC-163501, is shown to be a potent antagonist of L-glutamine in the majority of the reactions entailing amidotransfer by the crude supernatant enzymes of fetal rat liver as well as by several purified bacterial enzymes. Since critical steps in the biosynthesis of purines, pyrimidines, and L-asparagine have all been shown to be interrupted by the drug in vitro, it is reasonable to speculate that a similar interruption in vivo might help to explain the mechanism by which the drug lyses tumor cells. Consonant with this conclusion are the microfluorometric analyses which demonstrate that NSC-163501 interrupts the passage of cells from the G_1/S boundary or inhibits the synthesis of DNA in early S phase, a time when the need for nucleic acid precursors is intense. That L-glutamine can moderate such cytotoxicity under certain conditions indicates that this amide and NSC-163501 are competing for the same catalytic sites. It is relevant to point out that the structure of NSC-163501, while cyclic, bears an unmistakable resemblance to the structure of CONV, another potent antagonist of L-glutamine (fig 2). Thus, the isoxazoline oxygen can be envisioned as analogous to the ketone of CONV, and in both compounds the reactive chlorine atom is roughly equidistant from the primary amino function, which we have found to be an important binding site for inhibitors of L-asparagine biosynthesis. In addition, in vitro, both molecules selectively interrupt the biosynthesis of L-asparagine when L-glutamine, but not ammonia, is a substrate. Nevertheless, in vivo, their behavior is distinctive: CONV is only a feeble inhibitor of L-asparagine biosynthesis in L5178Y/AR leukemia after an ip dose of 100 mg/kg, whereas NSC-163501 exerts significant inhibition at doses 20 times lower than this, despite its greater molecular weight. It would appear that physiologic factors, such as uptake or metabolism, have facilitated in vivo inhibition by NSC-163501 while minimizing the efficacy of CONV (3).

One unexplained result of the present series of experiments is the elevation of L-threonine observed in pancreatic extracts from mice treated with NSC-163501. This finding raises the possibility that L-glutamine is somehow involved in the intermediary metabolism of L-threonine. Alternatively, it can be speculated that NSC-163501 interrupts protein synthesis, perhaps by restricting the availability of L-asparagine, and that other amino acids accumulate as a consequence of this action.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3
Azaserine: NSC-742; CAS reg. No. 115-02-6; serine, diazoacetate (ester), L-

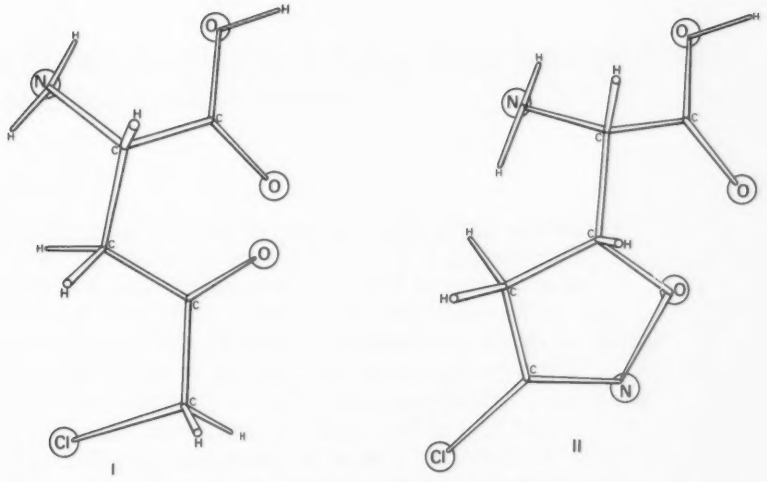

FIGURE 2.—Structures of CONV (I) and NSC-163501 (II).

CONV: NSC-124412; CAS reg. No. 15148-06-8; 5-chloro-4-oxo-L-norvaline; levulinic acid, 2-amino-5-chloro-, hydrochloride, L-

DON: NSC-7365; norleucine, 6-diazo-5-oxo-, L-

γ-Glutamyl hydrazide: NSC-7786; CAS reg. No. 1820-73-1; glutamic acid, 5-hydrazide, L-

NSC-163501: L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid

NSC-176324: (αS,4S,5R)-α-amino-3-chloro-4-hydroxy-4,-5-dihydro-5-isoxazoleacetic acid

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Survival and Phase Sensitivity of HeLa Cells Treated With Dianhydrogalactitol (NSC-132313)[1,2,3]

I. Pályi [4,5,6]

SUMMARY

The effect of dianhydrogalactitol on survival and phase sensitivity of the cell cycle of HeLa cells was studied. In survival experiments no cells survived when the drug was added and left in contact with the cells at or above a dose of 0.7 μg/ml. Cells synchronized by the mitotic-selection method showed changing sensitivity to the drug during the cell cycle. G_1, late-S, and M phases were more sensitive to dianhydrogalactitol than the early-S and G_2 phases. The DNA synthetic phase was divided into an early low sensitive and a late highly sensitive stage. The different sensitivity of mid-G_1 and early-S phases was further established in separate survival experiments. Cells treated in G_2 phase entered M phase in a manner similar to untreated control cells, while the progress of cells in S or G_1 phase during treatment was delayed significantly. Lack of phase specificity of this drug is consistent with that of other alkylating agents. Changing sensitivity during some phases of the cell cycle proves that some metabolic events are especially sensitive to this particular drug.

[Cancer Chemother Rep 59:493–499, 1975]

Cytostatic hexitols such as mannitol mustard, mannitol myleran, dibromomannitol, and dibromodulcitol[7] have been shown to induce cytomorphologic alterations typical for alkylating agents (1–6). These changes were characterized by cell enlargement and multinucleation, various mitotic abnormalities appearing after mitotic delay, increase of nuclear DNA content, and chromosome aberrations (7,8). These observations prompted us to investigate how these drugs influence cellular kinetics in vitro. It was shown that both mannitol mustard and dibromodulcitol lengthened the cell-cycle time of HeLa and Chinese hamster cells (8,9). This effect was due partly to the depression of the DNA synthetic rate and the prolongation of the S phase.

Dianhydrogalactitol, synthesized by L. Institóris et al (10) at the Chinoin Pharmaceutical and Chemical Works, Ltd., Budapest, Hungary, proved to be a potent tumor inhibitor as shown by Németh et al (11). It seems to be the most

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[2] The work reported in this paper was undertaken during the tenure of an American Cancer Society-Eleanor Roosevelt International Cancer Fellowship awarded by the International Union Against Cancer.

[3] Dianhydrogalactitol: CAS reg. No. 23261-20-3; 1,2:5,6-dianhydrogalactitol.

[4] Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, England. *Present address:* Research Institute of Oncopathology, Budapest, Hungary.

[5] The author wishes to thank Prof. L. G. Lajtha for his criticism of the manuscript and Drs. A. H. W. Nias and Margaret Fox for valuable discussions.

[6] *Reprint requests to:* Dr. I. Pályi, Research Institute of Oncopathology, Budapest XII, Ráth Gy. u. 7, Hungary.

[7] Mannitol mustard: NSC-9698; CAS reg. No. 551-74-6; mannitol, 1,6-bis[(2-chloroethyl)amino]-1,6-dideoxy-, dihydrochloride, D-; Degranol.

Mannitol myleran: NSC-37538; mannitol, 1,6-dimethanesulfonate, D-.

Dibromomannitol: NSC-94100; CAS reg. No. 488-41-5; 1,6-dibromo-1,6-dideoxy-D-mannitol; Myelobromol.

Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-; Elobromol.

toxic drug for cells in culture among the cytostatic hexitol derivatives studied so far by us. This work was intended to determine survival, phase sensitivity, and progress of HeLa cells treated with dianhydrogalactitol.

MATERIALS AND METHODS

The technique for in vitro culture of HeLa cells was the same as previously described by Nias (12). A line of HeLa cells cloned in the Paterson Laboratories was grown in 4-oz medical flat bottles inoculated with 1×10^5 cells in 10 ml of medium (Dulbecco's modification of Eagle's medium with 10% human serum). Cells were maintained in logarithmic growth by subculture twice weekly. Under these conditions the population doubling time was 25–27 hours. The drug was dissolved in ice-cold saline and sterilized by passage through a membrane filter (pore size, 0.22μ; Millipore Filter Corp.). To avoid its decomposition in long-term experiments where treatment was fractionated throughout a day, freshly made solution of dianhydrogalactitol was distributed into ampules, frozen very quickly, and kept at −20°C until use.

Dose-survival curves were determined according to the method of Puck and Marcus (13). One thousand cells were inoculated into each of a series of 4-oz flasks and incubated for 10 days. Different doses of dianhydrogalactitol were given to 10-ml cultures prior to the 10-day incubation by addition of 0.05–0.2 ml of drug solution. For calculation of the percentage of survivors, the control samples were normalized to 100% survival.

For labeled mitosis experiments, cells were seeded onto coverslips in Leighton tubes. The next day the cells were pulse-labeled with 2 microcuries/ml of ^{3}H-thymidine (specific activity, 5 curies/mmol; Radiochemical Centre, Amersham, England) for 15 minutes. Cold thymidine was then added to the cultures in a concentration of 10 μg/ml. Half the cultures were treated with a dose of 50 μg/ml of dianhydrogalactitol for 15 minutes. Samples of treated and untreated control cultures were fixed at intervals, and labeled and unlabeled mitoses were counted in orcein-stained preparations after radioautography. Preparations were immersed in Ilford L4 nuclear emulsion and exposed for 9 days.

Synchronized M-phase cells were obtained for phase sensitivity studies by the mitotic-selection method of Terasima and Tolmach (14). The degree of synchrony was estimated by determining: (*a*) the mitotic index (MI) immediately after collecting mitotic cells, (*b*) the average cellular multiplicity at hourly intervals, and (*c*) the labeling index (LI) at hourly intervals. Cellular multiplicity was determined by counting the number of single and paired cells in populations plated into Falcon plastic flasks. Such counts were made on separate flasks fixed at 1-hour intervals. For LI determination, cells were pulse-labeled with ^{3}H-thymidine at 2-hour intervals after synchronization and were then fixed and prepared for radioautography. Survival of synchronized cells was determined similarly to that of asynchronous cells. After inoculation, the M-phase cells were divided and attached to the glass within 1 hour. The first group of cultures was treated with the drug 2 hours after inoculation. Plating efficiency of untreated control cultures was 80%–85%.

For phase-sensitivity experiments, a short treatment schedule was used by adding 25 μg/ml of dianhydrogalactitol to the cells for 15 minutes, which allowed approximately 10% survival in asynchronous cultures. Specific examination of the M and G_2 phases was achieved by treating asynchronous monolayer cultures with dianhydrogalactitol for 15 minutes and shaking off and collecting mitotic cells immediately and 1, 3, and 5 hours respectively after treatment. These cells were also used for survival studies.

RESULTS

The effect of dianhydrogalactitol on survival of logarithmically growing asynchronous HeLa cells is shown in figure 1. Colonies containing fewer than 50 cells were not counted. Plating efficiency of the untreated control cultures was between 70% and 80%. The drug was left in contact with the cells for the 10-day period. No surviving cells were found at or above 0.7 μg/ml of drug concentration. Survival of cells after short-term exposure is shown in figure 2.

All manipulations of phase-sensitivity experi-

ments were made in a thermostatically controlled room at 37°C. At zero time, the MI of synchronized cells was 84%. Figure 3 shows the average cellular multiplicity and the LI at different times after synchronization. A total of 1.8 cells per colony was the average during the first 18 hours, which meant that 80% of the colonies contained two daughter cells originating from the mitotic cell. LIs indicated a low percentage of S-phase cells during the first 8 hours, ie, in G_1 phase. The labeling pattern shows that the length of G_1 phase was 10 hours and that of S phase was 9 hours. The length of the G_2 phase (5.5 hours) was determined by subtracting M + G_1 + S from cell-cycle time since the population tends to lose synchrony after 20–22 hours.

The survival level had not changed considerably during the first 10 hours, ie, during G_1 phase (fig 4). By the 12th hour, cell resistance increased abruptly followed by a gradual decrease until about the 20th hour, which corresponds to the S–G_2 transition. The curve then rose again, indicating an increasing tendency of cell resistance during G_2 phase. Specific examination of G_2 and M phases showed that M cells were much less resistant than late-G_2 cells. Earlier stages of the G_2 phase showed decreasing resistance (fig 5).

A difference in sensitivity to dianhydrogalactitol between mid-G_1 and early-S cells was confirmed in a separate experiment. Synchronized cells were treated with different doses of the drug at the fifth and 12th hour after synchronization. We have found a difference in sensitiv-

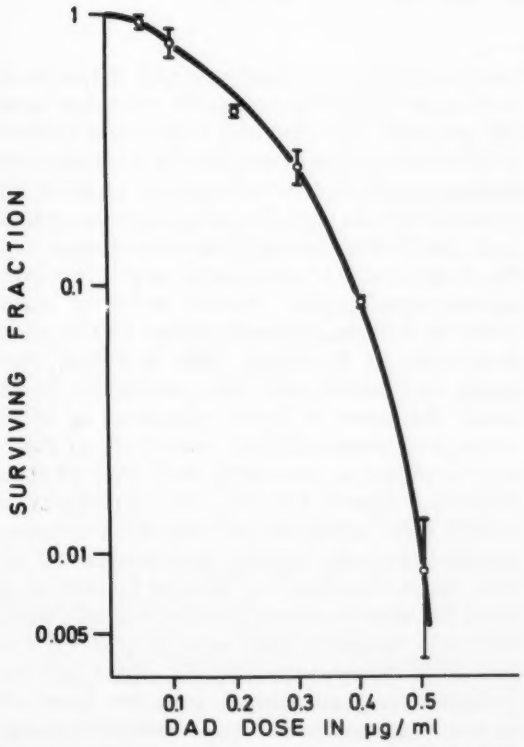

FIGURE 1.—Survival curve of asynchronous HeLa cells treated with dianhydrogalactitol (DAD). Exposure time: 10 days.

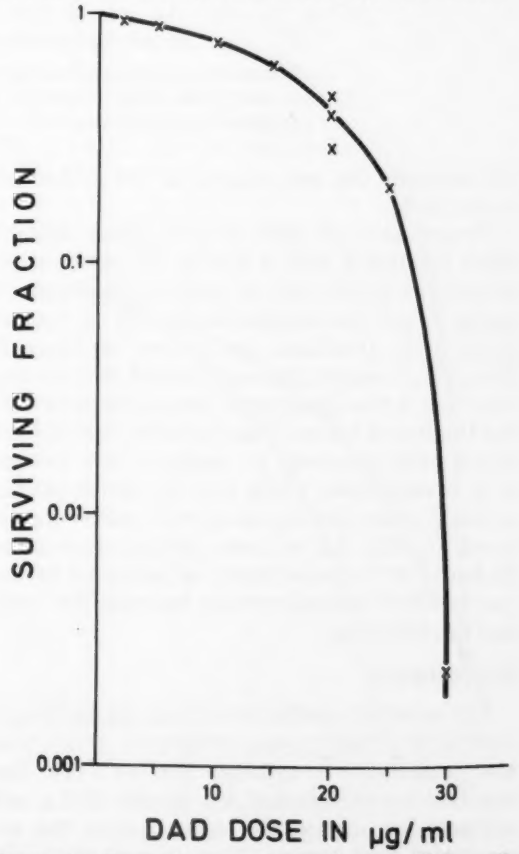

FIGURE 2.—Survival curve of asynchronous HeLa cells treated with dianhydrogalactitol (DAD). Exposure time: 15 mins.

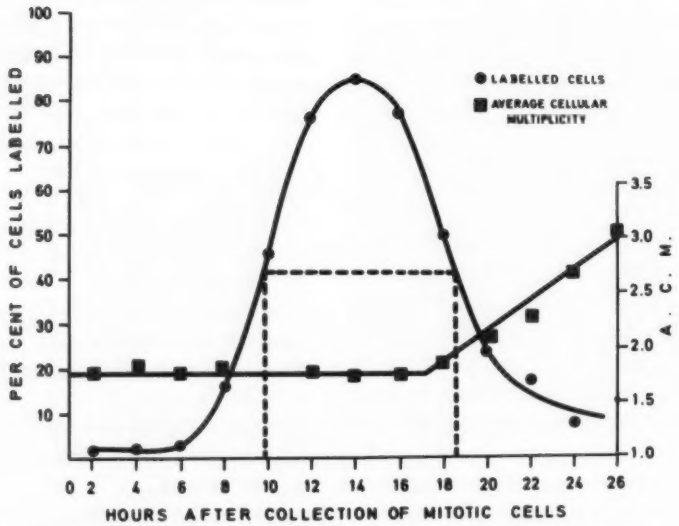

FIGURE 3.—Average cellular multiplicity (ACM) and LI of synchronized HeLa cells. Cells were labeled with 2 microcuries/ml of ^{3}H-thymidine for 15 mins.

ity between the two stages of the HeLa cell cycle (fig 6).

Progression of cells into M phase after a short treatment with a dose of 50 μg/ml of dianhydrogalactitol for 15 minutes is shown in figure 7, and the changes in the MI at various times after treatment are shown in figure 8. The rate of accumulation of labeled mitoses was identical in the control and treated cultures during the first 8 hours. This indicated that G_2 and late-S cells appeared to progress into mitosis at a normal rate, while cells in earlier stages of the S phase during drug treatment were delayed. G_1 cells did not enter mitosis during the 24 hours of the experiment as indicated by the nearly 100% labeled mitoses between the tenth and the 24th hour.

DISCUSSION

Cell-survival studies show that dianhydrogalactitol is a highly toxic substance, which was also established in cytologic studies (11). The survival curve obtained by treated HeLa cell cultures has a shoulder region before the exponential part begins. This means that cells accumulate damage which ultimately leads to a lethal effect (15). This type of curve is characteristic of radiation treatment and of treatment with most alkylating agents. On the other hand, the presence of a shoulder region can indicate a threshold, in the sense that, at a certain concentration, an insufficient amount of drug can penetrate to the sensitive site to produce an effect. Lack of plateau of the curve means that the drug is not a phase-specific agent. However, age-response studies showed different sensitivity of cells in different phases of the cycle. Sensitivity of M and G_1 cells is higher compared to early-S cells. The sensitivity is actually the same or lower compared to late-S or early-G_2 phases. Higher sensitivity of the M and G_1 phases is consistent with that of other alkylating agents (16,17). This sensitivity of mitotic cells cannot be detected with cytomorphologic methods. Mitotic abnormalities or mitotic death was observed only on the second or third day after treatment. Cells in late-G_2 phase were less sensitive than cells in late-S phase, and their progression into M phase did not change after treatment. G_2 cells are known to be the least sensitive to most cytostatic drugs.

The phase of DNA synthesis consists of an early low sensitive and a late highly sensitive period, with respect to its response to dianhy-

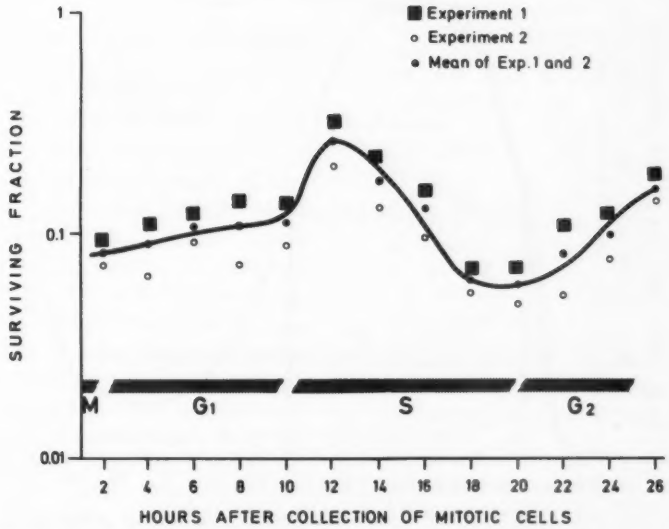

FIGURE 4.—Survival of synchronized HeLa cells after a short treatment with dianhydrogalactitol at different times during the cell cycle. Drug solution (0.1 ml) was added to 10 ml of medium to reach a 25-μg/ml end-concentration. After 15 mins the medium was replaced by drug-free medium.

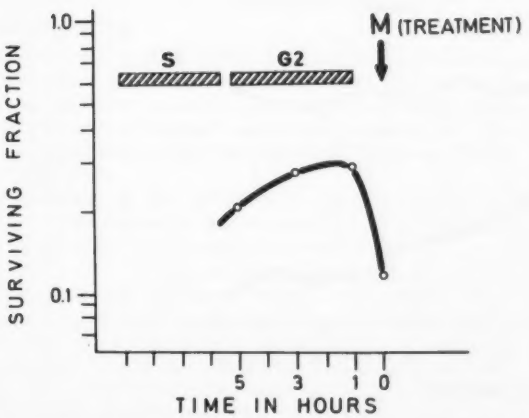

FIGURE 5.—Survival of HeLa cells treated in M and G_2 phases respectively. Asynchronous monolayer culture was treated with 25 μg/ml of dianhydrogalactitol for 15 mins. The medium was then replaced by drug-free medium, and mitotic cells were shaken off, collected, and plated for survival immediately and 1, 3, and 5 hrs after treatment.

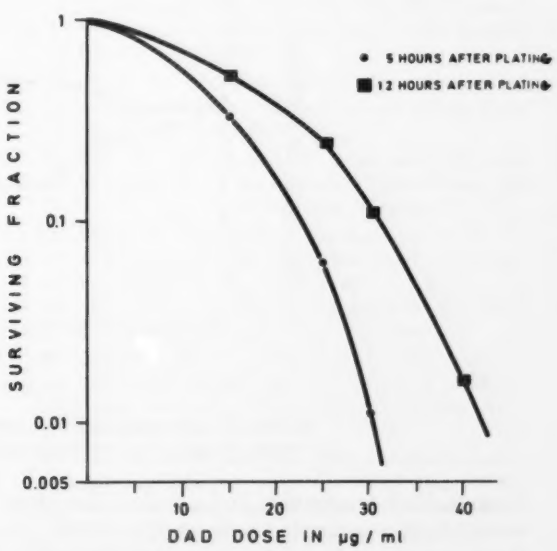

FIGURE 6.—Survival of synchronized HeLa cells treated with dianhydrogalactitol 5 and 12 hrs after synchronization and plating in mid-G_1 and early-S phases. Exposure time: 15 mins.

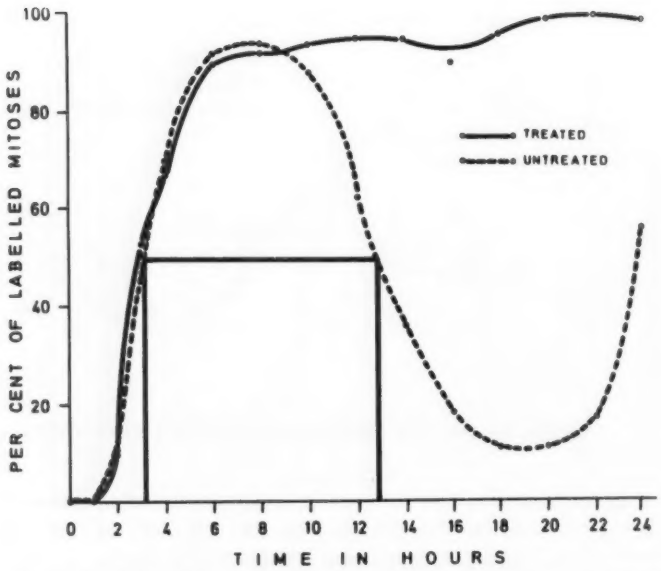

FIGURE 7.—Labeled mitotic wave at different times after a 15-min treatment of asynchronous HeLa cells with 50 μg/ml of dianhydrogalactitol. After exposure of cultures to 2 microcuries/ml of ^{3}H-thymidine for 15 mins, the medium was replaced by fresh medium containing 10 μg/ml of unlabeled thymidine.

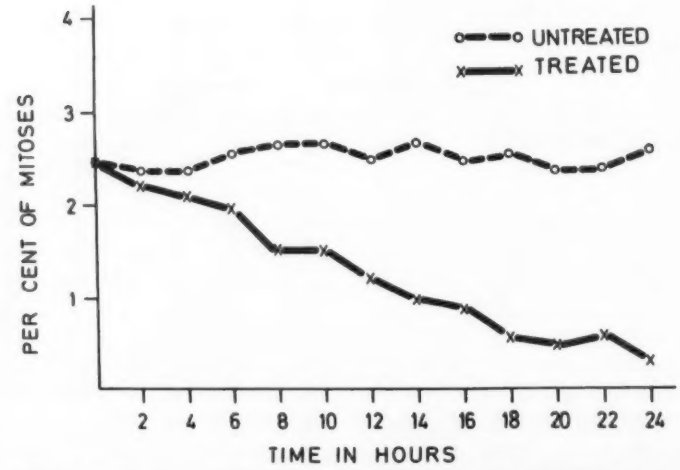

FIGURE 8.—Changes of MI in asynchronous HeLa cell cultures treated with 50 μg/ml of dianhydrogalactitol. Exposure time: 15 mins.

drogalactitol treatment. HeLa cells, like other transcription-controlled cells, require RNA synthesis mostly in early-S phase (18). The third part of the DNA synthetic phase is very sensitive to some RNA and protein inhibitors such as actinomycin D (NSC-3053) and *p*-fluorophenylalanine (NSC-71433) (19). Kozuka and Moore (20) found that the first part of the S phase was characterized by a low rate and the second part by a high rate of ^{3}H-thymidine uptake. The same was true for L cells (21). Lowered sensitivity of cells in early-S phase can be

correlated with the lower rate of thymidine incorporation.

G_2 cells progressed into M phase at the same rate as did the untreated cells. It seems probable that biochemical events which are important for the preparation for mitosis have been finished or are less sensitive to dianhydrogalactitol in the G_2 phase than those in other phases of the cell cycle. It should be emphasized that for compounds like dianhydrogalactitol, which has a relatively long half-life (L. Institóris, personal communication; I. Pályi, unpublished observations), it is difficult to determine precisely the effect in a synchronous cell population. Therefore, studies are planned to complement these observations with work on labeled compounds, to check drug binding and penetration into the cells, and to see how easy it is to wash the drug off the cells.

Our previous studies with other cytostatic hexitols showed alterations in the length of the cell-cycle time due to the prolongation of the S phase (8,9). In the present study with dianhydrogalactitol, we could detect differences in sensitivity during the S phase. Subdivision of the S phase of HeLa cells may contribute to a better understanding of the mode of action of dianhydrogalactitol at the subcellular level.

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Comparative Studies of Cyclocytidine (NSC-145668) and Cytosine Arabinoside (NSC-63878) in Mice [1,2,3]

Robert H. Liss [4] and Gary L. Nell [5,6]

SUMMARY

The distribution of cyclocytidine and cytosine arabinoside has been studied in normal BDF_1 mice and in mice bearing 6-day solid L1210 lymphocytic leukemia by whole-body radioautography, bioassay, and radiochemical techniques. Radioactivity was widely distributed throughout the tissues between 15 minutes and 12 hours after a single intravenous dose of either cyclocytidine-2-^{14}C or cytosine arabinoside-2-^{14}C. Whole-body radioautograms demonstrated that for most tissues, cytosine arabinoside-derived ^{14}C was uniformly excreted by 48 hours; cyclocytidine-derived ^{14}C, however, was localized in certain tissues as early as 15 minutes after drug administration and was retained in these sites for 48 hours. Depot loci of ^{14}C included salivary and adrenal glands, fat, cardiac muscle, gastrointestinal tract, and L1210 tumor. The distribution and persistence of cyclocytidine-derived radioactivity is consistent with other reports of toxicity induced by the drug in these tissues. Radiochromatography and bioassay data from BDF_1 mice dosed intraperitoneally with cyclocytidine demonstrated that 65%–95% of the ^{14}C-radioactivity in a number of tissues was the parent compound itself. Thus, cyclocytidine contributed in large measure to the generation of the radioautograms. This study demonstrates that the retention of cyclocytidine in body tissues may serve to effect the sustained release of the deaminase-resistant chemotherapeutic drug from these depot sites and thus prolong cytotoxic levels of drug in tumor tissue.

[Cancer Chemother Rep 59:501–513, 1975]

The clinical utility of cytosine arabinoside, a chemotherapeutic agent active against a number of acute leukemias and lymphomas, is compromised by the deamination of cytosine arabinoside to uracil arabinoside (araU), an inactive metabolite (1,2). The distribution and activity of the nucleoside deaminase are relatively high in man (3) in whom cytosine

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[2] Supported by contracts NO1-CM-33727, NO1-CM-33707, and NO1-CM-43753 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Cyclocytidine: CAS reg. No. 10212-25-6; 6*H*-furo-[2′,3′:4,5]oxazolo-[3,2-*a*]pyrimidine-2-methanol, 2,3,3*a*,-9*a*-tetrahydro-3-hydroxy-6-imino-, monohydrochloride; O^2,O^2-anhydro-1-β-furanosylcytosine; cycloC.

Cytosine arabinoside: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; araC; Cytosar.

Cyclocytidine (as the hydrochloride salt) and cytosine arabinoside were supplied by Drug Research and Development, DCT, NCI.

[4] Arthur D. Little, Inc., Cambridge, Mass.

[5] The Upjohn Company, Kalamazoo, Mich.

[6] *Reprint requests to:* Robert H. Liss, PhD, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass 02140.

arabinoside exhibits a comparatively short blood and tissue half-life (2,4). These observations have led to the study of cyclocytidine (5–7), a potent antitumor agent which is less easily deaminated than cytosine arabinoside (8). A number of studies have been published on the chemotherapeutic activity and physiologic disposition of cyclocytidine and cytosine arabinoside (9–11) in which the resistance of cyclocytidine to deamination is discussed (8,9).

The present collaborative investigation was undertaken to compare the whole-body radioautographic distribution of cytosine arabinoside and cyclocytidine with companion data on tissue, blood, and excretory levels of the compounds, in order to assess the interrelationships among drug distribution, deaminase activity, and chemotherapy.

MATERIALS AND METHODS

For whole-body radioautography, BDF_1 mice weighing 20 g were implanted subcutaneously in the axillary region with 40-mg fragments of solid L1210 lymphocytic leukemia. After 6 days, normal and tumor-bearing mice were given single intravenous (iv) injections via the caudal vein of either cytosine arabinoside-2-^{14}C or cyclocytidine-2-^{14}C in saline. Both drugs were administered at doses of 100 mg/kg and 100 microcurie/kg.

Groups of mice were frozen in a dry-ice/hexane mixture at 15 and 30 minutes and at 1, 3, 6, 12, 24, and 48 hours after dosage. All animals were subsequently processed for whole-body radioautography (12).

For the physiologic disposition studies, female BDF_1 mice were given single intraperitoneal (ip) injections of either cytosine arabinoside-2-^{14}C or cyclocytidine-2-^{14}C (as the hydrochloride salts) at doses of 500 mg/kg and 10–60 microcurie/kg. At various times, mice (four/time) were killed, heparinized blood was obtained and pooled, and plasma and cells were separated by centrifugation (at 4°C). Tissues (brain, kidney, small intestine, liver, lung, and spleen) were excised, rinsed (ice-cold 0.9% NaCl), weighed, pooled, and homogenized (about 5%–20% wt/vol) in ice-cold 0.2 N perchloric acid (PCA). Homogenates were centrifuged and supernatants (extracts) were separated from the pellets. After washing (0.9% NaCl), blood cells were also extracted with 0.2 N PCA. Radioactivity of plasma, tissue, and blood-cell PCA extracts and of pellets remaining after PCA extraction was determined using tissue solubilizers (where necessary) and liquid scintillation techniques. In all cases, acid-insoluble radioactivity (pellets) was less than 10% of that found in supernatant extract (homogenate). Total radioactivity in the supernatant extract and insoluble residue was expressed as μg of cyclocytidine or cytosine arabinoside equivalents per g of tissue (or ml of plasma) in figure 1 and per ml of blood in figures 2 and 3.

Plasma macromolecules (proteins) were precipitated with 3 volumes of ethanol and radioactivity was determined by using liquid scintillation techniques.

The composition of plasma-ethanol supernatants (or concentrated samples therefrom) obtained from mice treated with ^{14}C-cyclocytidine was determined using thin-layer chromatography involving the following two systems:

System I—precoated silica gel F254 plates (Silplate F-52, 0.25 mm, Brinkmann) developed in a solvent consisting of acetone:water (92:8, vol/vol).

System II—precoated silica gel GF plates (Uniplate, 0.25 mm, Analtech, Inc.) developed in water.

The R_f values in Systems I and II respectively were: cytosine arabinoside (0.35, 0.85); cyclocytidine (0.25, 0.40); araU (0.65, 0.90); and cyclouridine (cycloU) (0.45, 0.75). After developing, plates were scraped in 1-cm sections, scrapings were added to counting vials containing 0.5 ml 0.5 N PCA, and after about 30 minutes counting solution was added and radioactivity was determined. In System II it was possible to readily distinguish between cyclocytidine, cycloU, and either cytosine arabinoside or araU. However, the resolution in this system was not sufficient to quantitate the relative amounts of the latter two materials. In none of the samples did the amount of radioactivity co-chromatographing with cycloU exceed 10% of the total, and these amounts were not considered significant. In System I it was possible to easily separate araU from the other likely

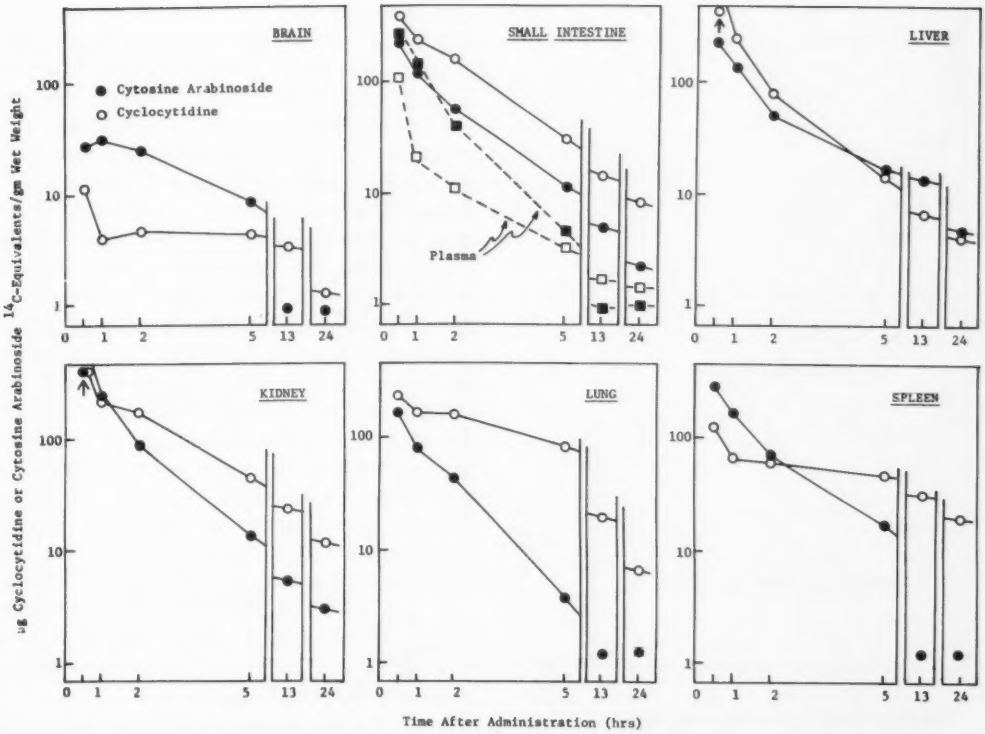

FIGURE 1.—Tissue deposition of total radioactivity after ip administration of 500 mg/kg of ^{14}C-cyclocytidine or ^{14}C-cytosine arabinoside to BDF$_1$ mice. ● = Cytosine arabinoside; ○ = cyclocytidine.

components and the proportion of cytosine arabinoside, cyclocytidine, and araU could thus be calculated. Radioactivity co-chromatographing with these three components accounted for over 90% of the radioactivity in all samples, and thus the possible presence of other materials was ignored in the calculations leading to the data of table 1. Composition of radioactivity in plasma-ethanol supernatants obtained from mice treated with ^{14}C-cytosine arabinoside was determined using previously described chromatography systems (9). For plasmas obtained up to 7 hours after administration of ^{14}C-cytosine arabinoside and up to 2 hours after administration of ^{14}C-cyclocytidine, over 90% of plasma radioactivity was recovered in the ethanol supernatant. However, in samples obtained at longer times after treatment, recoveries decreased (to as low as about 20% at 24 hours after administration). Attempts to identify the nature of the radioactivity precipitated with the plasma macromolecules have not yet been successful. Total plasma radioactivities in table 1 are based on recoverable radioactivities. Because of the low levels of radioactivity in plasmas obtained at 5 hours or more after administration of either agent, it was only possible to roughly estimate the percent of composition at 5 and 7 hours. Very low levels of radioactivity in the plasmas 12 and 24 hours after administration precluded estimates of composition.

The composition of the excreted urine was determined radiochromatographically (using the chromatography systems described above) and by a differential bioassay technique (13) capable of the estimation of the relative amounts of cyclocytidine and cytosine arabinoside in biologic fluids.

In a separate experiment, the amounts of

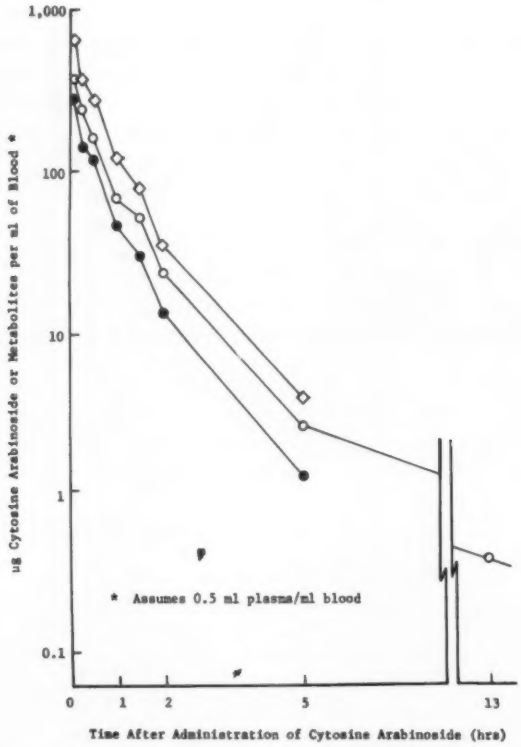

FIGURE 2.—Blood radioactivity levels after ip administration of ^{14}C-cytosine arabinoside (500 mg/kg) to BDF$_1$ mice. ◇ = Total (plasma + cells); ○ = plasma; ● = cells.

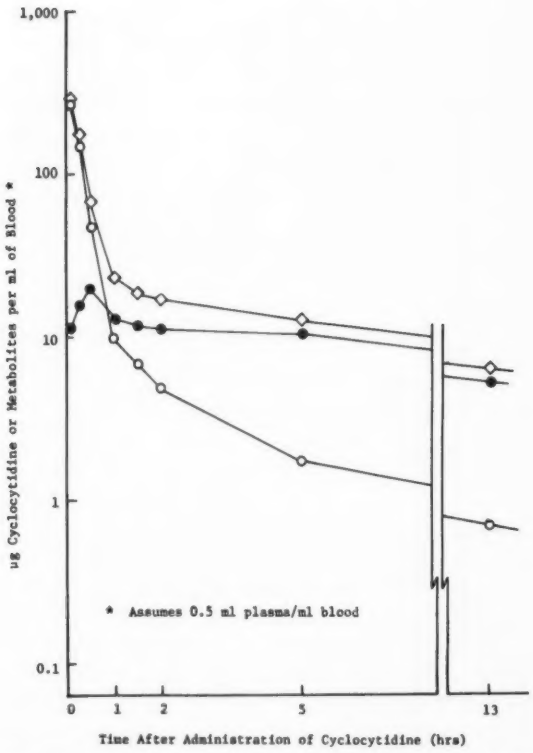

FIGURE 3.—Blood radioactivity levels after ip administration of ^{14}C-cyclocytidine (500 mg/kg) to BDF$_1$ mice. ◇ = Total (plasma + cells); ○ = plasma; ● = cells.

TABLE 1.—Radiochromatography of plasma obtained from mice treated ip with 500 mg/kg of ^{14}C-cyclocytidine or ^{14}C-cytosine arabinoside

	Administration of—						
	^{14}C-cyclocytidine				^{14}C-cytosine arabinoside		
		Composition (%)				Composition (%)	
Time (hrs) after dosage	Total mg/ml of plasma*	Cyclocytidine	Cytosine arabinoside	AraU	Total mg/ml of plasma	Cytosine arabinoside	AraU
0.08	778	81	18	1	716	87	13
0.25	301	80	18	2	639	75	25
0.5	133	60	26	11	287	94	6
1	24.6	44	36	20	158	56	44
2	13.8	23	47	30	61	36	64
5	2.2	(35)	(35)	(40)	4.7	(50)	(50)
7	1.6	(25)	(38)	(38)	3.4	(50)	(50)
13	0.46	—	—	—	0.24	—	—
24	0.19	—	—	—	0.14	—	—

*Based on mol wt of agent administered and on radioactivity recoverable after precipitation of plasma macromolecules.

cytosine arabinoside and cyclocytidine in mice tissues 1 hour after treatment with cyclocytidine (500 mg/kg, ip) were estimated by using the differential bioassay. This assay is not capable, however, of detecting nucleotide metabolites of cytosine arabinoside or cyclocytidine or of detecting the potential deamination products, araU or cycloU. The detection microorganism used for this assay (*Streptococcus faecalis*, actinobolin resistant) is actually sensitive only to cytosine arabinoside (and not to cyclocytidine). However, when samples are treated briefly under basic conditions, cyclocytidine can quantitatively be converted into cytosine arabinoside. Thus, sample assays before and after base treatment allow estimation of the quantities of both cytosine arabinoside and cyclocytidine. Cytosine arabinoside nucleotides are not converted to cytosine arabinoside under the conditions employed. In these latter studies, tissues were freeze-thawed and homogenized in acetic acid (1 M, 4°C) and were assayed after lyophilization and reconstitution in water. Appropriate control tissue homogenates (from untreated mice) were processed identically and were employed for preparation of standard curves.

RESULTS

Excretion Studies

The cumulative urinary excretion of ^{14}C-radioactivity after administration of either ^{14}C-cytosine arabinoside or ^{14}C-cyclocytidine to BDF_1 mice is shown as a function of time in figure 4A. After administration of cyclocytidine, excretion of radioactivity is considerably slower and less complete than that observed after cytosine arabinoside is administered. In the first 7 hours after administration of cytosine arabinoside, 73% of the initial dose of radioactivity was excreted, whereas only 42% was excreted in the same time period after dosage with cyclocytidine.

The composition of excreted urine, determined by radiochromatography, is shown in figures 4B and 4C. Only cytosine arabinoside and araU were formed in the urine of mice treated with ^{14}C-cytosine arabinoside. Although cytosine arabinoside predominated in the first

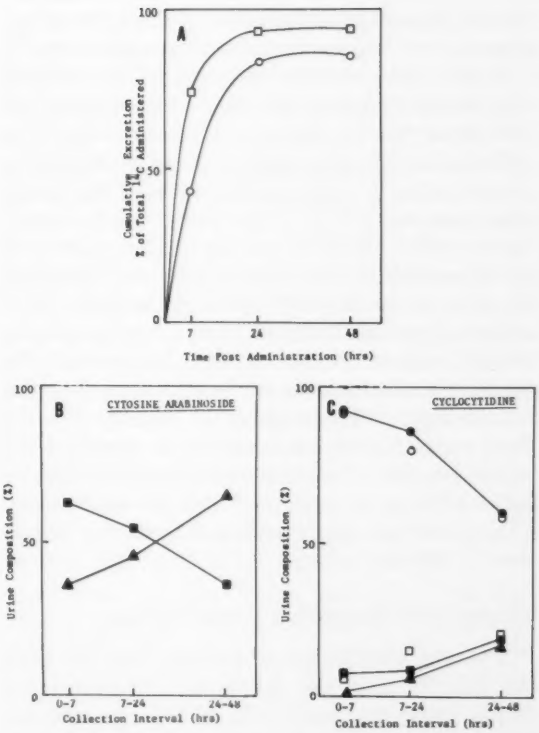

FIGURE 4.—Urinary excretion after ip administration of 500 mg/kg of ^{14}C-cytosine arabinoside or ^{14}C-cyclocytidine to BDF_1 mice. A. □ = Cytosine arabinoside and ○ = cyclocytidine. B. ■ = Cytosine arabinoside and ▲ = AraU. C. ○ = Cyclocytidine bioassay; ● = cyclocytidine; ■ = cytosine arabinoside; ▲ = AraU; and □ = cytosine arabinoside bioassay.

7 hours, araU was the major component in the urine obtained in the interval from 24 to 48 hours after drug administration. At all times when ^{14}C-cyclocytidine was administered, the predominant urine component was cyclocytidine itself. At later times, however, cytosine arabinoside and araU components did show significant increases, so that each of these two metabolites accounted for approximately 20% of the radioactivity in the urine collected 24–48 hours after drug dosage. The relative concentration of cyclocytidine and cytosine arabinoside in the urine obtained from mice treated with cyclocytidine was also determined using a differential bioassay technique and these results are shown in figure 4C. These results are in

good agreement with those obtained by radiochromatography; however, the araU content (which cannot be detected by bioassay) was assumed from the radiochromatographic data.

After the administration of ^{14}C-cytosine arabinoside, 94% of the administered dose was recovered in the urine in 48 hours: 58% as cytosine arabinoside and 36% as araU. After dosage with ^{14}C-cyclocytidine, 83% of the initial dose was recovered in the urine for the same time period: 74% as cyclocytidine, 6.5% as cytosine arabinoside, and 2.5% as araU. During the first 24 hours, 0.2% of administered radioactivity was recovered in fecal extracts of mice treated with ^{14}C-cyclocytidine. Because of the possible contamination of feces with urine, the significance of the apparently slightly greater fecal excretion of cyclocytidine is doubtful. In other studies (unpublished results, Upjohn laboratories), excretion of ^{14}C via respiratory CO_2 accounted for less than 5% of the initial dose of radioactivity.

Plasma and Blood-Cell Level Studies

The concentrations of radioactivity in both the plasma and cell fractions of centrifuged whole blood obtained from BDF_1 mice treated with ^{14}C-cytosine arabinoside and ^{14}C-cyclocytidine, respectively, are shown in figures 2 and 3. The data are expressed as μg of administered drug equivalent or metabolite/ml of blood. The disappearance of total radioactivity in the blood of mice treated with ^{14}C-cytosine arabinoside was approximately first order for at least the 5-hour administration. The half-life for disappearance of radioactivity over the first 2 hours was 27 minutes. The disappearance of total blood radioactivity after the administration of cyclocytidine approximated first-order kinetics for only 1 hour after administration; the half-life was determined as 13 minutes. After this time, the half-life for disappearance increased to approximately 8–9 hours. The ratio of radioactivity in plasma and blood cells remained rather constant for the first 5 hours after administration of ^{14}C-cytosine arabinoside, during which period 54%–69% of the initial dose of ^{14}C was in plasma. However, when ^{14}C-cyclocytidine was administered, the percent of total blood radioactivity found in the blood cells increased from approximately 4% at 5 minutes to 90% 5–13 hours after administration. Thirteen hours after administration, blood cells contained approximately 5.5 μg of cyclocytidine or metabolites of equivalent molecular weight/ml of blood.

The composition of radioactivity observed in the plasma of mice treated with cyclocytidine is shown as a function of time in table 1. The total concentrations were obtained after correcting for radioactivity recoverable after precipitation of plasma proteins and other macromolecules with ethanol. Because of the low radioactivity levels observed, the values listed for 5- and 7-hour postadministration must be considered merely as rough estimates. Thirteen and 24 hours after administration, the extremely low radioactivity levels obtained precluded radiochromatographic determination. With both compounds, the relative concentration of araU increased with time, so that 1 hour or more after drug dosage, levels of araU and cytosine arabinoside were comparable in plasma.

Tissue Level Studies

The deposition of ^{14}C-radioactivity derived from cyclocytidine and cytosine arabinoside is shown as a function of time for several tissues in figure 1. In the brain and plasma, cytosine arabinoside equivalents were higher than cyclocytidine equivalents for the first 5 hours; thereafter, cyclocytidine equivalents were more concentrated in these compartments. For other tissues, including lung, kidney, spleen, and small intestines, cyclocytidine generally exhibited greater tissue deposition through 24 hours. Both drugs had almost equivalent concentrations in the liver throughout the study. Complementary whole-body radioautographic observations follow, which compare the tissue deposition of cytosine arabinoside- and cyclocytidine-derived ^{14}C over a 48-hour period.

The composition of cyclocytidine-derived radioactivity in several tissues was estimated in a separate experiment. A differential bioassay, capable of determining both cytosine arabinoside and cyclocytidine levels, was employed with tissues obtained 1 hour after ip administration of 500 mg/kg of ^{14}C-cyclocytidine. These data

appear in table 2. The percents of radioactivity which can be attributed to unchanged drug (cyclocytidine) for several tissues are: heart, 79%; small intestine, 85%; kidney, 75%; liver, 75%; lung, 95%; salivary gland, 95%; spleen, 65%; and thymus, 74%. Cytosine arabinoside, as measured by this technique, was present to an extent not greater than 10% in any of the tissues. Note that this technique does not detect araU, cycloU, or nucleotides of cytosine arabinoside, and presumably would not detect nucleotides of cyclocytidine. More rigorous chromatographic studies would be required to establish the levels of these metabolites.

Whole-Body Radioautography Studies

Nervous system.—Significant low radioautogram density was observed in the brain and spinal cord 1 hour after the administration of ^{14}C-cytosine arabinoside. However, after cyclocytidine dosage, density above background was first apparent between 3 and 6 hours and was no longer evident after 12 hours. Results of the tissue deposition studies (fig 1) are in general agreement with the whole-body radioautograms (figs 5A and 5B, 6A and 6B, and 7A and 7B) and indicate that radioactivity in the central nervous system was higher for the first 5 hours after administration of cytosine arabinoside than after dosage with cyclocytidine. However, cytosine arabinoside-derived radioautogram density was not detected in central nervous system tissue after 12 hours while levels of cyclocytidine-derived density persisted through 48 hours.

Lymphatic organs.—Radioactivity in splenic tissue remained significantly above background through 48 hours after administration of ^{14}C-cyclocytidine and through 3 hours after dosage with ^{14}C-cytosine arabinoside. Between 3 and 48 hours, cytosine arabinoside-derived radioautogram density fell to levels slightly above background. The white pulp was more dense than the red in all observations after cyclocytidine administration; no such difference in ^{14}C deposition was observed with cytosine arabinoside (figs 5B and 6A). After administration of either agent, radioautogram density in the thymus was above background at all times studied, rising to moderate levels by 6 hours.

Pancreas.—The pancreas exhibited a low level of radioautographic density at all time periods after administration of either drug (fig 5B). Tissue density was comparable to the spleen, muscle, liver, and L1210 tumor.

Adrenal glands.—Drug deposition in the adrenal glands of mice treated with cyclocytidine increased to a moderate density by 1 hour and persisted through 12 hours. After cytosine arabinoside was administered, adrenal gland radioactivity was only slightly above background through 48 hours.

Salivary glands.—The salivary glands of mice treated with ^{14}C-cyclocytidine showed extremely high radioautographic density at all time periods from 15 minutes to 48 hours. ^{14}C uptake in the parotid and submaxillary glands was

TABLE 2.—Tissue radioactivity and drug levels 1 hr after administration of ^{14}C-cyclocytidine to BDF$_1$ mice

Tissue	Cyclocytidine (μg)/g wet weight of tissue		Cyclocytidine: % of total radioactivity
	^{14}C drug equivalents*	Bioassay†	
Heart	122	96	79
Intestine	207	176	85
Kidney	188	141	75
Liver	226	170	75
Lung	192	182	95
Salivary gland	346	329	95
Spleen	68	44	65
Thymus	78	58	74

*± 10%.
†± 20%.

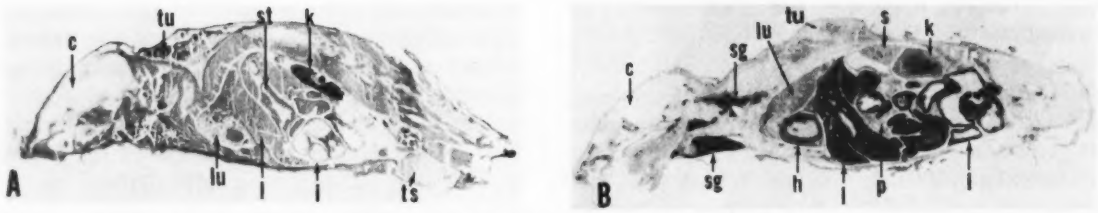

FIGURE 5.—Whole-body radioautograms of BDF_1 mice bearing L1210 solid tumors. These radioautograms are representative of rodents killed between 15 min and 1 hr after iv administration of (A) ^{14}C-cytosine arabinoside (100 mg/kg; 100 microcurie/kg) and (B) ^{14}C-cyclocytidine (100 mg/kg; 100 microcurie/kg). c = Cerebrum; h = heart; i = intestine; k = kidney; l = liver; lu = lung; p = pancreas; s = spleen; sg = salivary gland; st = stomach; ts = testis; tu = tumor.

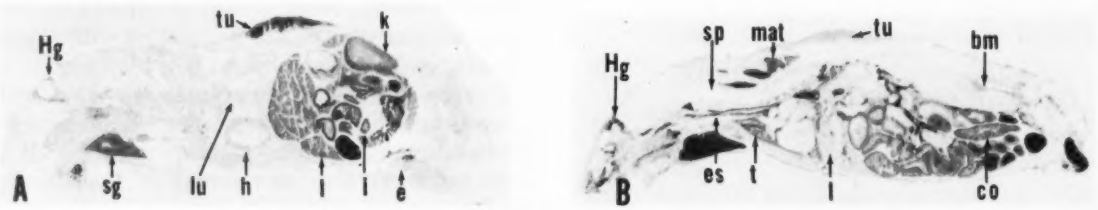

FIGURE 6.—Whole-body radioautograms of BDF_1 mice bearing L1210 solid tumors. These radioautograms are representative of rodents killed between 3 and 12 hrs after iv administration of (A) ^{14}C-cytosine arabinoside (100 mg/kg; 100 microcurie/kg) and (B) ^{14}C-cyclocytidine (100 mg/kg; 100 microcurie/kg). bm = Bone marrow; co = colon; e = epididymis; es = esophagus; h = heart; Hg = Harderian gland; i = intestine; k = kidney; l = liver; lu = lung; mat = multilocular adipose tissue; sg = salivary gland; sp = spinal cord; t = thymus; tu = tumor.

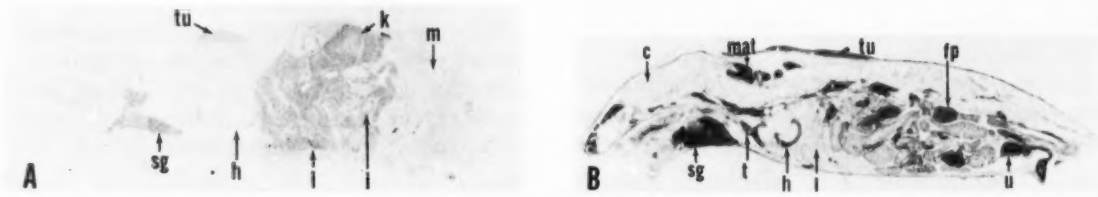

FIGURE 7.—Whole-body radioautograms of BDF_1 mice bearing L1210 solid tumors. These radioautograms are representative of rodents killed between 24 and 48 hrs after iv administration of (A) ^{14}C-cytosine arabinoside (100 mg/kg; 100 microcurie/kg) and (B) ^{14}C-cyclocytidine (100 mg/kg; 100 microcurie/kg). c = Cerebrum; fp = fecal pellet; h = heart; i = intestine; k = kidney; l = liver; m = muscle; mat = multilocular adipose tissue; sg = salivary gland; t = thymus; tu = tumor; u = urinary bladder.

greater than in the major sublingual gland. In contrast, after administration of ^{14}C-cytosine arabinoside, the salivary glands were only slightly above background density through 6 hours.

Digestive system.—The linings of the stomach and intestines were prominent at all time periods tested after the administration of ^{14}C-cyclocytidine. Radioautographic density in the liver appeared slightly higher after ^{14}C-cyclocytidine than after the administration of ^{14}C-cytosine arabinoside. One hour after dosing with ^{14}C-cyclocytidine, areas of increased density were evident in the hepatic parenchyma, presumably in ducts carrying ^{14}C-enriched bile to the gallbladder. The density of the gallbladder increased to moderate levels 1 hour after cyclocytidine administration and persisted for

approximately 6 hours. After administration of ^{14}C-cytosine arabinoside, the gallbladder and the lining of the gastrointestinal tract showed radioautographic density only slightly above background. Radioautogram density was more pronounced in the small intestine after ^{14}C-cyclocytidine than after ^{14}C-cytosine arabinoside through 48 hours. No discernible differences in the ^{14}C content of the hepatic parenchyma were resolved.

Respiratory system.—Radioactivity was only slightly above background in nasal mucosa, trachea, and lung parenchyma 1 hour after the administration of cytosine arabinoside; at the same time, lung tissue of mice treated with ^{14}C-cyclocytidine showed moderate density. Comparable density was observed by 3 and 6 hours after administration of ^{14}C-cytosine arabinoside. Nasal mucosa and trachea appeared more dense than lung parenchyma, possibly due to the secretions from the very dense salivary glands.

Urinary system.—Both agents showed similar radioautographic density in the kidney. The renal pelvis, ureter, and urinary bladder contained urine in which a high level of radioactivity was evident. These observations were consistent with renal excretion being the principal route of elimination of the drug equivalents.

Hard tissues.—With both agents, no radioautograms of the bones or teeth were obtained for any time during the study. ^{14}C deposition in the bone marrow of the spinal column, sternum, and long bones increased to somewhat higher levels after the administration of ^{14}C-cyclocytidine than after the administration of ^{14}C-cytosine arabinoside.

Muscle and fat.—After administration of ^{14}C-cyclocytidine, radioautograms of the heart muscle were more dense than those of the skeletal muscle at all time periods; after ^{14}C-cytosine arabinoside, radioactivity in both of these tissues was slightly above background. With both agents, fatty tissue maintained a low level density throughout the experiment. A notable exception was multilocular adipose tissue (brown fat) in the neck of mice treated with ^{14}C-cyclocytidine. As early as 15 minutes after dosing, the tissue exhibited a moderate level of ^{14}C. The level of ^{14}C-radioactivity peaked at 1 hour and persisted for 3 hours after dosing. Thereafter, drug-derived density continued at a moderate but significant level through 48 hours.

Tumor tissue.—After administration of ^{14}C-cyclocytidine, tumor tissue exhibited a radioactive density slightly above background through 48 hours. In contrast, after ^{14}C-cytosine arabinoside, tumor tissue radioactivity was moderately dense through 12 hours after dosing. The cortex of the solid L1210 tumor was generally more dense than the necrotic central area, after dosage with either cytosine arabinoside or cyclocytidine.

DISCUSSION

Clinical observations on the rapid conversion of cytosine arabinoside by cytidine deaminase to the inactive metabolite araU prompted investigations which led to the study of cyclocytidine, a compound less easily deaminated (14). The significance of this chemical modification of cytosine arabinoside was reflected in radioautograms of BDF$_1$ mice dosed with either radiolabeled cytosine arabinoside or cyclocytidine; these radioautograms revealed remarkable differences in the absorption, distribution, tissue and organ retention, and excretion of ^{14}C-radioactivity derived from the compounds.

Radioautograms of mice prepared 15 and 30 minutes after administration of either ^{14}C-cytosine arabinoside or ^{14}C-cyclocytidine demonstrated rapid absorption and extensive whole-body distribution of drug equivalents. However, the wide homogeneous distribution of cytosine arabinoside-derived radioautogram density contrasted with the specific localizations of cyclocytidine equivalents in a number of tissues and organs including salivary glands, liver, lining of the gastrointestinal tract, and cardiac muscle. Thus, even in this short time after drug administration, significant differences in whole-body distribution of the two chemotherapeutic agents were already apparent (fig 5A). Greater vascular density at this time period was observed in radioautograms of mice dosed with cytosine arabinoside than in litter mates treated with cyclocytidine. Companion radiochemical data also indicated a higher level of cytosine

arabinoside drug equivalents in the circulating plasma (fig 2) while other studies demonstrated a biologic half-life of 80 minutes for cytosine arabinoside and 37 minutes for cyclocytidine in rodents (15).

Differential retention and excretion of cytosine arabinoside- and cyclocytidine-^{14}C equivalents in rodent tissues were observed radioautographically between 1 and 12 hours after drug administration. Peak density in radioautograms of cytosine arabinoside-dosed mice was observed after 3 and through 12 hours. Most dense were the gallbladder, liver, and gastrointestinal contents, presumably indicative of biliary secretion of drug equivalents into the intestine. Enterohepatic recirculation of ^{14}C was suggested by the recovery of 94% of the administered dose of ^{14}C-cytosine arabinoside in the urine in 48 hours and essentially none in feces. Urinary excretion and enterohepatic recirculation were similarly indicated for cyclocytidine.

An apparent uniform excretion of cytosine arabinoside-derived ^{14}C-radioactivity from most body tissues was demonstrated by the progressive loss of radioautogram density through 48 hours. Only in gastrointestinal lining, salivary glands, kidney, liver, heart, and tumor were tissues somewhat more dense than the general musculature. The relatively rapid and homogeneous excretion of cytosine arabinoside-derived ^{14}C was consistent with observations that the compound is most active when administered in courses of multiple closely spaced doses or constant infusion (15).

Significant concentrations of cyclocytidine-derived ^{14}C persisted in a number of loci through 48 hours, long after the 6-hour time period when peak density was observed in whole-body radioautograms. The deposition and retention of cyclocytidine equivalents in fat, adrenal, salivary, lymphatic, and cardiac tissue were impressive and suggest that the relatively low toxicity, high antitumor activity, and relative absence of schedule dependence (6) correlate not only with the resistance of the compound to cytidine deaminase activity but also with the release of the depot form of the parent compound from sequestered tissue sites over a prolonged period of time. It would be interesting to determine the distribution of pyrimidine nucleoside deaminase activity in these murine tissues which have been shown in this study to be depot sites for cyclocytidine. Unfortunately, previous observations on the distribution of deaminase activity in the mouse did not include these organs (3).

A limitation inherent in whole-body radioautography is the identification of the ^{14}C-labeled parent compound and metabolites; the value of a collaborative study such as this investigation lies in companion resolution, by radiochromatography and bioassay, of the ^{14}C-moieties which generated radioautograms. The physiologic disposition studies in this investigation have shown that approximately 75% of the administered dose of cyclocytidine was recovered in the urine within 24 hours as intact drug. In addition, cyclocytidine was found to contribute 65%–95% of the drug-derived radioactivity in tissues 1 hour after administration of cyclocytidine. The lack of significant ($<$ 10%) levels of cytosine arabinoside in those tissues is also in agreement with other data obtained in rats (16). Those investigators also were unable to detect significant levels of araU. Since little cytosine arabinoside or araU is found in the urine excreted in 24 hours, one might reasonably expect that cyclocytidine remains the major component in tissues throughout this period.

Hypersalivation, salivary gland discomfort, and gastrointestinal toxicity including mild inhibition of the spontaneous gastrointestinal motility are toxic side effects reported to follow cyclocytidine administration (15,17). However, the cumulative toxicity of cyclocytidine has been found to be less prominent than that of cytosine arabinoside (18). Such pathologic changes are consistent with the localization and persistence of radioactivity for 48 hours in the mucosal layer of the gastrointestinal tract of rodents, and with the persistence of cyclocytidine in the small intestine of mice at significant concentrations through 24 hours (fig 1). Other studies on the distribution of cyclocytidine- and cytidine analog-derived ^{14}C in mice (19) did not identify or quantitate the radiolabeled moieties in murine tissues, including those for which toxicity has been noted.

The c[illegible] of the solid L1210 tumor contained

significant concentrations of cytosine arabinoside-derived ^{14}C-radioactivity which persisted through 48 hours. Cyclocytidine radioautogram density in tumor tissue generally paralleled that observed for cytosine arabinoside. In both instances, an unequal distribution of radioactivity between the inner and outer layers of the tumor mass was probably the result of poor vascularization at the center of the advanced tumor. By 48 hours, the radioautographic density of both cytosine arabinoside- and cyclocytidine-treated L1210 tumors was slightly above background (table 3). The radioautographic observations suggest that the differences in antitumor activity between cytosine arabinoside and cyclocytidine can be explained in part by the hypothesis that the latter is a depot form of the active moiety. The steady release of cyclocytidine over a long period may promote high relatively constant therapeutic concentrations of the drug in tumor tissue. On the other hand, since cytosine arabinoside is an S-phase specific agent with both a short plasma half-life (9) and relatively rapid excretion from most murine body tissues (including L1210 tumor), chemotherapeutic concentrations of drug over time may not be obtained.

The whole-body radioautographic observations in this investigation have shown that a major difference between cytosine arabinoside and cyclocytidine distribution in mice is the localization and persistence of the latter in specific tissues and organs. Companion radiochemical studies have demonstrated that these radioautographic densities were generated in large measure by the parent compound, cyclocytidine. These results suggest that a sustained release of deaminase-resistant cyclocytidine from tissue depot sites may yield high, prolonged concentrations of cytotoxic levels of cytosine arabinoside in tumor cells, and may explain the marked improvement observed when cyclocytidine is substituted for cytosine arabinoside in a therapeutic regimen (6).

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TABLE 3.—Summary of distribution of cytosine arabinoside- and cyclocytidine-derived ^{14}C-radioactivity in normal and tumor-bearing BDF_1 mice

Tissue	Radioautographic density*— Normal mice— time (hrs) killed after dosage						Tumor-bearing mice— time (hrs) killed after dosage							
	0.5	1	3	6	12	24	0.25	0.5	1	3	6	12	24	48
							Cyclocytidine							
Skeletal muscle	2	2	2	2	1	1	1	1	1	1	1	1	1	1
Central nervous system	1	2	2	1	1	0	1	1	1	2	1	1	0	0
Lung	2	2	2	1	1	0	1	1	1	1	1	1	1	0
Heart	2	2	2	2	1	1	1	1	1	1	1	1	1	1
Thymus	2	3	4	4	3	2	1	1	1	3	3	3	2	1
Liver	2	2	2	2	1	1	1	1	1	1	2	2	2	1
Spleen	2	2	2	1	1	1	1	1	1	1	1	1	1	1
Pancreas	2	2	2	1	1	1	1	1	1	1	1	1	2	1
Renal cortex	2	2	2	2	1	1	1	1	1	2	2	2	2	1
Renal medulla	2	2	2	2	1	1	1	1	1	2	2	2	2	1
Adrenal cortex	1	2	2	2	2	2	1	1	1	2	2	—	2	2
Adrenal medulla	1	2	1	1	1	1	1	1	1	1	1	—	1	1
Lining of the gastrointestinal tract	2	2	2	2	2	2	1	1	1	1	1	2	2	1
Gastrointestinal contents	0	3	4	4	4	2	0	0	1	1	3	3	3	0
Bone marrow	1	2	2	2	1	1	1	1	1	1	1	1	1	1
Bone and teeth	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Skin	2	2	2	2	1	1	1	1	1	1	1	1	1	1
Blood	2	2	1	0	0	0	1	1	1	1	0	0	0	0
Testes	2	2	2	2	1	1	1	1	1	2	1	1	1	1
Multilocular adipose tissue	1	2	1	1	1	1	1	1	1	1	1	1	1	1
Salivary glands	1	1	2	2	2	2	1	1	1	1	2	2	2	2
Harderian gland	1	1	1	1	2	2	1	1	1	1	1	2	1	1
Intraorbital lacrimal gland	1	1	1	1	2	2	1	1	1	1	1	2	1	1
Gallbladder	1	3	4	4	—	0	—	1	2	4	4	—	—	0
Retina	2	3	3	3	3	2	1	1	2	2	2	2	1	1
Tumor							1	1	2	2	3	3	2	1
							Cyclocytidine							
Skeletal muscle	2	2	1	1	1	1	1	1	1	1	1	1	1	1
Central nervous system	0	1	1	1	1	1	0	0	0	1	1	1	1	1
Lung	2	2	2	2	1	1	1	1	2	2	2	1	1	1
Heart	1	2	2	2	3	3	1	2	2	2	2	3	2	2
Thymus	1	2	2	2	3	2	0	1	2	3	3	2	2	2
Liver	2	2	1	1	1	1	3	2	2	1	1	1	1	1
Spleen	2	2	2	1	1	1	1	1	1	1	1	1	1	1
Pancreas	1	1	1	1	1	1	1	1	1	1	2	1	1	1
Renal cortex	1	2	2	2	1	1	2	1	1	1	1	1	1	1
Renal medulla	1	2	2	2	1	1	2	1	1	1	1	1	1	1
Adrenal cortex	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Adrenal medulla	2	2	4	4	4	4	1	2	3	4	4	4	4	4
Lining of the gastrointestinal tract	3	3	2	1	1	1	3	3	4	2	2	2	1	1
Gastrointestinal contents	0	3	3	3	4	3	0	0	2	3	3	3	3	3
Bone marrow	1	2	2	1	1	1	1	1	1	1	1	1	1	1
Bone and teeth	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Skin	2	2	3	3	3	3	2	2	2	2	3	3	3	2
Blood	1	1	0	0	0	0	1	1	1	1	1	0	0	0
Retina	1	1	2	1	1	1	0	1	1	1	1	2	2	1

TABLE 3.—Summary of distribution of cytosine arabinoside- and cyclocytidine-derived ^{14}C-radioactivity in normal and tumor-bearing BDF_1 mice—Continued

Tissue	Radioautographic density*— Normal mice— time (hrs) killed after dosage						Tumor-bearing mice— time (hrs) killed after dosage							
	0.5	1	3	6	12	24	0.25	0.5	1	3	6	12	24	48
							Cyclocytidine							
Multilocular adipose tissue	2	3	3	3	3	3	1	2	3	3	4	4	4	2
Salivary glands	3	4	4	4	4	4	4	4	4	4	4	4	4	2
Harderian gland	2	3	3	3	3	1	1	1	2	2	2	2	1	1
Intraorbital lacrimal gland	2	3	3	3	3	1	1	1	2	2	2	2	1	1
Gallbladder	1	1	1	3	—	0	1	1	3	2	1	—	0	0
Lymph nodes and thoracic duct	2	4	4	4	4	4	3	3	1	4	4	4	4	2
Tumor							1	1	4	2	2	2	2	1

*0 = Density at background; 1 = density slightly above background; 2 = density significantly above background; 3 = moderate density; and 4 = extreme density.

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Microbiologic Assay for Cytosine Arabinoside (NSC-63878): The Use of a Mutant of *Streptococcus faecium* var. *durans* Resistant to Methotrexate (NSC-740) and 6-Mercaptopurine (NSC-755)[1,2,3,4]

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SUMMARY

Cytosine arabinoside has been shown to be effective in the treatment of acute leukemias and lymphomas, especially in combination with other established anticancer agents such as methotrexate, 6-mercaptopurine, and 6-thioguanine. A microbiologic assay capable of detecting cytosine arabinoside in the presence of such anticancer agents in body fluids has been developed using a strain of *Streptococcus faecium* var. *durans* resistant to methotrexate and 6-mercaptopurine; the assay can also be used to determine cytosine arabinoside concentrations in the presence of 6-thioguanine. In view of the present trend toward drug combinations, including those using tetrahydrouridine, in the chemotherapy for human neoplasms, the proposed assay method will be valuable in cytosine arabinoside distribution studies.

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[4] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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One of the antitumor pyrimidine nucleosides, cytosine arabinoside, has an established place in clinical chemotherapy for acute leukemias and lymphomas (1–4). Since its accepted use as an antineoplastic agent, there has been a need for a rapid, sensitive, and inexpensive method for the determination of free cytosine arabinoside in tissues and body fluids. Methods employing tissue culture techniques with L1210 cells (5) and L cells (6) have been described. Because these methods are time consuming, Haňka et al (7) developed a microbiologic assay method as an improvement over the one described by Pittillo and Hunt (8), using an actinobolin-resistant strain of *Streptococcus faecalis* ATCC 8043 (SF/ACB). The method described by Haňka et al (7) detects cytosine arabinoside concentrations as low as 0.1 μg/ml in biologic fluids, a sensitivity quite adequate for some studies on the distribution of the drug in animals and man.

Recently, we carried out a survey of available streptococcal strains in our laboratory in an at-

tempt to find a strain more sensitive to cytosine arabinoside than SF/ACB, as well as a strain which is not only sensitive to this drug but is also resistant to other anticancer agents. Since cytosine arabinoside is used extensively in cancer chemotherapy in combination with other anticancer agents, an assay method that would determine drug concentrations in the presence of such agents and tetrahydrouridine, an inhibitor of deaminase (9), would be advantageous.

The present report describes a microbiologic assay procedure using a strain of *Streptococcus faecium* var. *durans* resistant to methotrexate (A) and 6-mercaptopurine (6-MP) (SF/A/MP) (10), which is not only sensitive but also can be used to determine cytosine arabinoside concentrations in the presence of methotrexate, 6-MP, and 6-thioguanine.

METHODS

The SF/A/MP culture was maintained as described by Hutchison (10) on Flynn-PP medium containing 1 ng/ml of folic acid and 300 μg/ml of 6-MP. The SF/ACB culture (obtained from Dr. R. F. Pittillo, Southern Research Institute, Birmingham, Ala) was maintained on complete Flynn medium (11) containing 1 ng/ml of folic acid and 10 μg/ml of actinobolin. *Streptococcus faecium* var. *durans* (SF/O) (10,12), the parent strain of both SF/ACB and SF/A/MP, is resistant to cytosine arabinoside and is used for comparison in the present study. SF/O was maintained on complete Flynn medium containing 1 ng/ml of folic acid (10). The complete folic acid assay medium of Flynn (11) and the Difco folic acid assay medium have very similar compositions and for all practical purposes are interchangeable. However, since Difco folic acid assay medium is a ready-mix powder, it does not have the versatility for single constituent manipulation as does the Flynn medium (11), which is prepared in the laboratory.

Determination of Folic Acid Requirement

Folic acid requirement in SF/ACB and SF/A/MP was compared with that of SF/O by conventional microbiologic vitamin assay technique, as described in detail by Hutchison (10), using concentrations of folic acid varying between 0.1 and 10 ng/ml in Flynn medium (11) with and without purines (adenine, guanine, and xanthine) and the pyrimidine, uracil.

Tube Assay of Cytosine Arabinoside

Inhibition of the growth of SF/ACB and SF/A/MP by cytosine arabinoside was compared with that of SF/O in liquid Flynn medium (11) with and without purines (adenine, guanine, and xanthine) and the pyrimidine, uracil, containing folic acid (1.0 ng/ml for all three cultures in Flynn medium and for SF/O and SF/ACB in Flynn-PP medium; 5 ng/ml for SF/A/MP in Flynn-PP medium) and various concentrations of cytosine arabinoside in the presence and absence of tetrahydrouridine (20 μg/ml). Inoculum of each of the organisms was prepared as follows: Each culture was grown overnight for 18 hours at 37°C in the liquid Flynn medium (11) supplemented with folic acid (1 ng/ml). The overnight cultures were washed twice with and resuspended in 0.85% saline to an optical density of 0.2 at 600 nm (approximately 6×10^7 cells/ml), as measured in a Bausch and Lomb "spectronic 20" spectrophotometer. The suspended culture was then further diluted one to six times and tubes were inoculated with 1 drop of this diluted culture into each tube (approximately 0.05 ml) using a 5-ml pipette. The growth of the organisms in the presence of different cytosine arabinoside concentrations was measured as absorbance (optical density) at 600 nm in a Beckman spectrophotometer (Model B) after incubation at 37°C for 18 hours. A plot of the optical density against the concentration of cytosine arabinoside was made, and the concentration of the drug that produced 50% inhibition of growth was determined.

Disc Assay of Cytosine Arabinoside

Both SF/ACB and SF/A/MP were also compared for sensitivity to cytosine arabinoside on agar plates using filter paper discs saturated with various concentrations of the drug. For the agar plate assay, we used Difco folic acid assay medium (Difco Laboratories, Detroit, Mich) as a matter of convenience instead of

Flynn medium, since the comparative studies with both media were similar. The assay medium contained 0.5 ng/ml of folic acid and 20 μg/ml of tetrahydrouridine, and the pH of the medium was adjusted to 6.0; however, pH adjustment was not necessary with SF/A/MP. Inoculum of each of the organisms was prepared as follows: Each culture was grown overnight for 18 hours at 37°C in the liquid Flynn medium (11) supplemented with only folic acid (1 ng/ml). The overnight cultures were washed twice with and resuspended in 0.85% saline to an optical density of 0.2 at 600 nm (approximately 6×10^7 cells/ml) as measured in a Bausch and Lomb "spectronic 20" spectrophotometer; 2.5 ml of the suspension was used as inoculum for 200 ml of medium containing 1.5% agar. Plates containing 10 ml each of the seeded agar medium were prepared and stored in the cold for at least 3–4 hours at 4°C prior to use in the assay. The solutions for the standard curves were made in saline at concentrations of 0.03, 0.05, 0.1, 0.3, 0.5, 1.0, 3.0, 5.0, and 10.0 μg/ml of cytosine arabinoside. Paper discs (Schleicher and Schull, No. 740-E) of 6.35- and 12.7-mm diameter were used for this comparative assay. They were saturated with 0.02 and 0.1 ml respectively of the standard cytosine arabinoside solutions. The plates were then incubated for 18 hours at 37°C. Zones of inhibition were measured in a zone reader (Fisher-Lilly Antibiotic Zone Reader, Fisher Scientific Co., Pittsburgh, Pa). Standard curves were plotted as averages of five-zone diameter measurements. The statistical variation in cytosine arabinoside determination using this method was within 5% ±0.052 (SD).

RESULTS AND DISCUSSION

Both SF/ACB and SF/A/MP required folic acid, and the quantitative requirement for half-maximum growth in complete Flynn medium (11) was similar to that of SF/O (table 1). However, SF/A/MP required almost 16 times more folic acid (5 ng/ml) than SF/ACB in the absence of purines (adenine, guanine, and xanthine) and the pyrimidine, uracil (Flynn-PP medium [10]). The folic acid requirement of SF/ACB remained the same (0.3 ng/ml) in the absence as well as in the presence of purines and uracil.

TABLE 1.—Quantitative folic acid requirements (ng/ml) for half-maximum growth

Organism	Folic acid assay medium	
	F*	F-PP†
SF/O	0.3	1.0
SF/ACB	0.3	0.3
SF/A/MP	0.3	5.0

*Folic acid-free medium of Flynn et al (11).

†Folic acid-free medium of Flynn et al (11) without purines (adenine, guanine, and xanthine) and without the pyrimidine, uracil.

The addition of 0.5 μg/ml of folic acid to the assay medium, as reported by Hanka and coworkers (7) who employed SF/ACB as the assay organism, was unnecessary. We observed that folic acid at 0.5 ng/ml in either Difco folic acid assay medium employed by them or in the Flynn medium (11) that we have occasionally used for both SF/ACB and SF/A/MP was sufficient to provide comparable results with disc assay for cytosine arabinoside determinations.

The cytosine arabinoside concentration for 50% inhibition of SF/A/MP in liquid medium was 0.3 μg/ml; however, 1.5 μg/ml of the drug was required to achieve similar effect in SF/ACB (table 2), with Flynn medium (11) containing 20 μg/ml of tetrahydrouridine. In the absence of tetrahydrouridine both SF/ACB and SF/A/MP were less sensitive, whereas SF/O was totally resistant to cytosine arabinoside in the presence as well as in the absence of tetrahydrouridine. Although SF/ACB was found to be more sensitive than SF/A/MP in Flynn medium devoid of purines (adenine, guanine, and xanthine) and the pyrimidine, uracil (table 2), the disc assay with SF/ACB in such medium was far from satisfactory. Thus, relatively higher sensitivity of SF/A/MP to cytosine arabinoside in the tube assay led us to compare the inhibitory effect of the drug using the disc-assay technique. In the disc assay, cytosine arabinoside not only produces sharper and more distinct zones of inhibition with SF/A/MP (fig 1) than with SF/ACB, but it was also detectable at a concentration of 0.03 μg/ml on the large discs (12.7 mm in diameter) and 0.05 μg/ml on the small discs (6.35 mm in diameter). Al-

though Difco folic acid assay medium adjusted to pH 6.0 improved the assay clarity and sensitivity to some extent with SF/ACB, it was found unnecessary to adjust the pH of the medium to pH 6.0 with SF/A/MP. Clear zones of inhibition as seen with SF/A/MP (fig 1) are easy to measure accurately on the zone reader, and clarity is retained at the lowest concentration of cytosine arabinoside on the standard curve. Zones obtained with SF/ACB (fig 1) are hazy and less distinct and require manual measurements; also, the lower concentrations of the cytosine arabinoside standard curve did not produce measurable zones. Similar difficulties in the measurement of the zones of inhibition obtained with SF/ACB have been reported by Neil et al (13). We confirm the findings of Haňka et al (7) that addition of tetrahydrouridine, a known inhibitor of deoxycytidine/cytosine arabinoside deaminase (9), increases the sensitivity of SF/ACB to the latter agent, and, in addition, we observed a similar effect on SF/A/MP (fig 2). Although lower amounts of tetrahydrouridine did affect the sensitivity of the assay, concentrations higher than 20 μg/ml did not significantly increase the sensitivity of either SF/ACB (7) or SF/A/MP (fig 2). Since tetrahydrouridine is not readily available in large quantities, we have used 20-μg/ml concentrations for all our assays. We compared the effect of heat sterilization with that of filter sterilization of tetrahydrouridine on the disc assay of cytosine arabinoside with SF/A/MP. Heat-sterilized tetrahydrouridine had an effect on increasing the potency of cytosine arabinoside similar to that of filter-sterilized tetrahydrouridine; therefore, for our experiments we have routinely added 20 μg/ml to the assay medium before autoclaving.

A most significant feature of an assay procedure using SF/A/MP is its sensitivity in the presence of methotrexate and 6-MP. Sensitivity of SF/A/MP to cytosine arabinoside was determined with solutions containing a mixture of cytosine arabinoside and different concentrations of methotrexate and/or 6-MP. SF/A/MP was found to be as sensitive to cytosine arabinoside in the presence of 10 μg/ml of methotrexate and 100 μg/ml of 6-MP alone or in combination as in the absence of these two drugs. SF/A/MP was also resistant to up to 100 μg/ml of 6-thioguanine. Haňka (14) reported that cytosine arabinoside can be assayed in the presence of 5 μg/ml of methotrexate when the assay medium is supplemented with 20 μg/ml of citrovorum factor for SF/ACB disc assay. Although cytosine arabinoside can be assayed in the presence of up to 10 μg/ml of methotrexate with SF/A/MP without any supplementation, by virtue of its being resistant to methotrexate, we compared SF/ACB and SF/A/MP assays for cytosine arabinoside determination in the presence of different concentrations of methotrexate in Difco folic acid assay medium in the absence (fig 3) and presence (fig 4) of 20 μg/ml of citrovorum factor. The results showed that cytosine arabinoside concentrations can be detected without any loss of sensitivity with SF/A/MP in the presence of up to 250 μg/ml of methotrexate when the assay medium is supplemented with

TABLE 2.—Growth inhibition of *Streptococcus faecalis* by cytosine arabinoside (μg/ml of cytosine arabinoside for 50% inhibition)

	F + FA medium*		F-PP + FA medium†	
Organism	Without THU‡	With THU	Without THU	With THU
SF/O	>1000.0	>1000.0	100.0	15.0
SF/ACB	15.0	1.5	3.5	0.6
SF/A/MP	4.2	0.3	5.0	1.3

*Folic acid-free medium of Flynn et al (11) supplemented with 1 ng/ml of folic acid.

†Folic acid-free medium of Flynn et al (11) without purines (adenine, guanine, and xanthine) and without the pyrimidine, uracil; supplemented with 1 ng/ml of folic acid for SF/O and SF/ACB and 5 ng/ml for SF/A/MP.

‡THU = tetrahydrouridine (20 μg/ml).

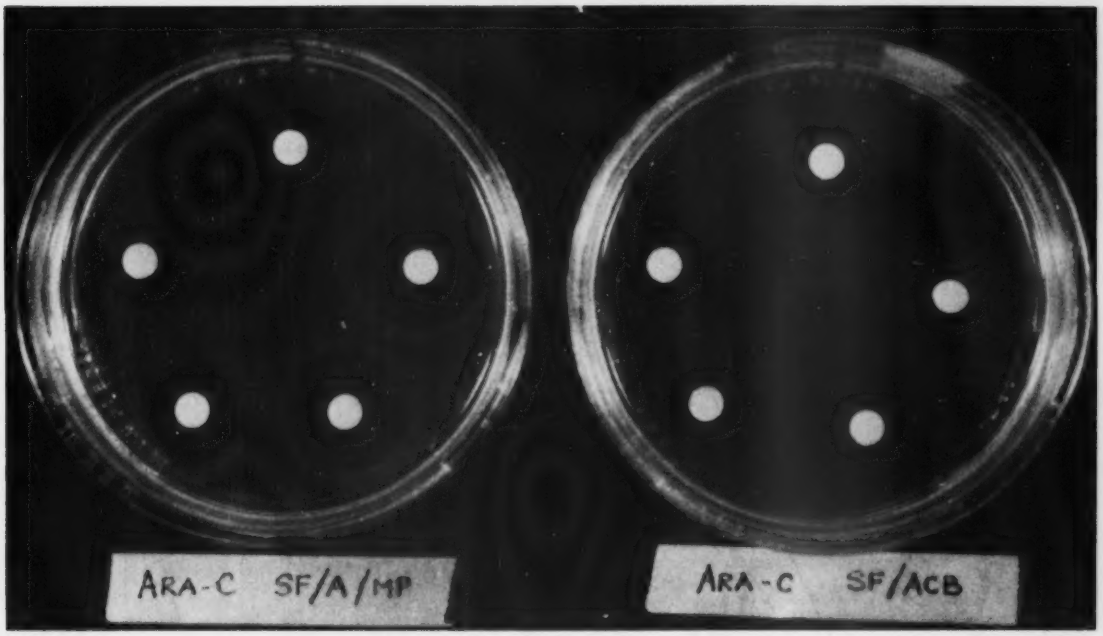

FIGURE 1.—Growth inhibition of SF/A/MP and SF/ACB by cytosine arabinoside (ara-C). Zones of inhibition with standard solutions of the drug containing (clockwise) 10, 5, 3, 1, and 0.5 μg/ml in 0.85% saline; 0.02 ml applied to each of the respective discs (6.35 mm in diameter). Assay medium was Flynn + folic acid (0.5 ng/ml) supplemented with 20 μg/ml of tetrahydrouridine.

20 μg/ml of citrovorum factor, whereas 50 μg/ml of methotrexate interfered with cytosine arabinoside assay using SF/ACB under similar conditions. Methotrexate at 25 μg/ml and higher concentrations interfered with the cytosine arabinoside assay when SF/A/MP was used without citrovorum factor in the medium. The disc-assay procedure presented here has been successfully adopted in our laboratory to determine cytosine arabinoside concentrations in the cerebrospinal fluid of cancer patients after intraventricular administration of the drug by the Ommaya reservoir (15). The recovery of cytosine arabinoside from serum, plasma, and cerebrospinal fluid was 98%–100%.

Cytosine arabinoside is usually administered in combination with other anticancer agents, such as 6-thioguanine, methotrexate, and 6-MP, and the microbiologic assay method proposed here would fulfill the requirements for a rapid analytical procedure for the determination of free cytosine arabinoside in the presence of these drugs.

LIST OF COMPOUNDS

Actinobolin: NSC-31083; isocoumarin, 4-(2-aminopropionamido)-3,4,4*a*,5,6,7-hexahydro-5,6,8-trihydroxy-3-methyl-

Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt; leucovorin; folinic acid

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytarabine; ara-C

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Tetrahydrouridine: NSC-112907; CAS reg. No. 5627-05-4; 2(1*H*)-pyrimidinone, tetrahydro-4-hydroxy-1-β-D-ribofuranosyl-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

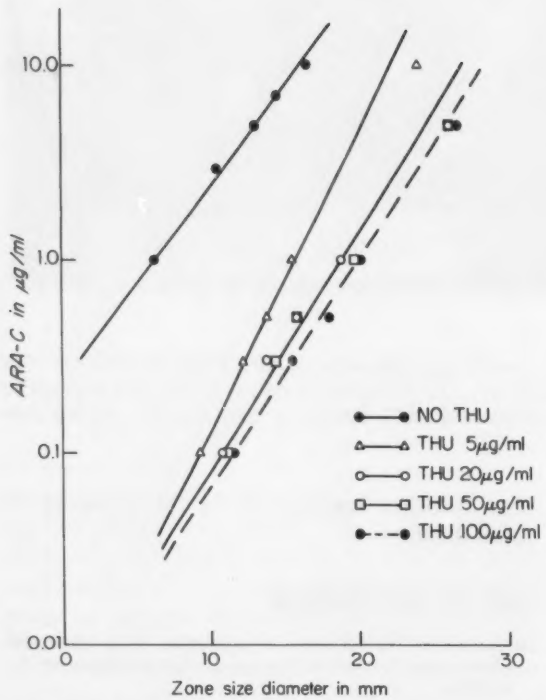

FIGURE 2.—Effect of tetrahydrouridine (THU) on growth inhibition of SF/A/MP by cytosine arabinoside (ara-C). Assay medium was that of Flynn et al (11), containing 0.5 ng/ml of folic acid.

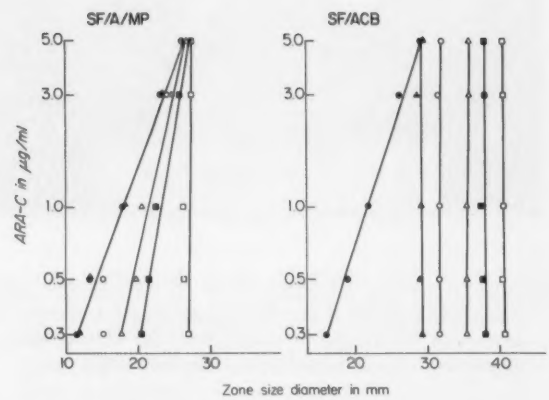

FIGURE 3.—Effect of methotrexate (MTX) on growth inhibition of SF/A/MP and SF/ACB by cytosine arabinoside (ara-C). Assay medium was Flynn + folic acid (0.5 ng/ml) supplemented with 20 μg/ml of tetrahydrouridine. ●———● = Ara-C standard solutions without MTX; ▲———▲ = ara-C standard solutions contained 2.5 μg/ml of MTX; ○———○ = ara-C standard solutions contained 5.0 μg/ml of MTX; △———△ = ara-C standard solutions contained 25.0 μg/ml of MTX; ■———■ = ara-C standard solutions contained 50.0 μg/ml of MTX; □———□ = ara-C standard solutions contained 250.0 μg/ml of MTX.

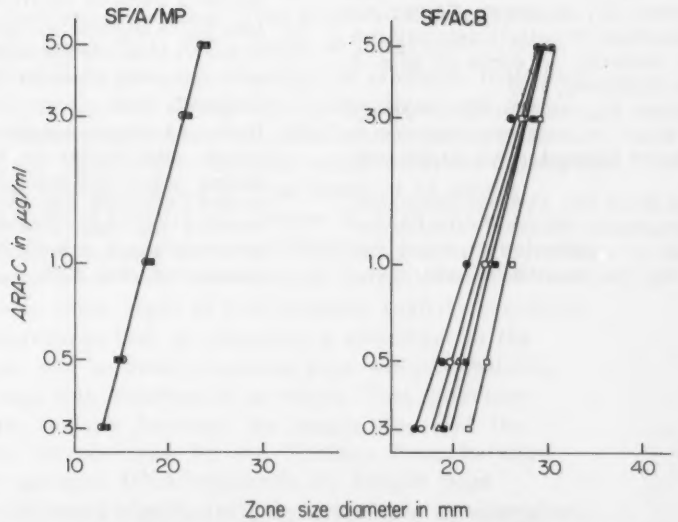

FIGURE 4.—Effect of methotrexate (MTX) on growth inhibition of SF/A/MP and SF/ACB by cytosine arabinoside (ara-C) in the presence of citrovorum factor. Assay medium was Flynn + folic acid (0.5 ng/ml) supplemented with 20 µg/ml of tetrahydrouridine and 20 µg/ml of citrovorum factor. ●——● = Ara-C standard solutions without MTX; ▲——▲ = ara-C standard solutions contained 2.5 µg/ml of MTX; ○——○ = ara-C standard solutions contained 5.0 µg/ml of MTX; △——△ = ara-C standard solutions contained 25.0 µg/ml of MTX; ■——■ = ara-C standard solutions contained 50.0 µg/ml of MTX; □——□ = ara-C standard solutions contained 250.0 µg/ml of MTX.

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Preclinical Studies With Triazinate (NSC-139105), an Antifolate Drug, in Beagle Dogs and Rhesus Monkeys [1,2,3]

Edward J. Gralla, Gerald L. Coleman, George W. Osbaldiston, Wendy L. Sawicki, Albert M. Jonas, and Joseph R. Bertino [4,5,6]

SUMMARY

The effects of the intravenous administration of triazinate by single and multiple injections were studied in beagle dogs and rhesus monkeys. In dogs, dose levels ranging from 0.3125 to 40 mg/kg were given either as single doses daily for 5 days, or once weekly for 6 weeks. The 5-day regimen was also studied in monkeys with dose levels from 2.5 to 40 mg/kg/day. Prominent drug-related and drug-dependent effects which appeared in both species were piloerection, muscular weakness, and respiratory difficulty which occurred during and immediately after the administration of dose levels of 10 mg/kg or greater. Gastrointestinal toxicity was severe in dogs but mild in monkeys. Lymphoid tissue toxicity was manifested by a circulating lymphopenia and localized cellular depletion in the germinal centers of lymphoid tissues. In dogs, signs of bone marrow toxicity consisted of a circulating neutropenia and, at necropsy, a reduction in the number of erythroid and myeloid elements plus megaloblastosis. Only the latter change was observed in monkeys. This difference in the hematopoietic toxicity between the beagle dog and the rhesus monkey was corroborated by the findings from in vitro studies with bone marrow. DNA synthesis in beagle bone marrow cells was depressed significantly by triazinate as compared with cells from rhesus marrow. A direct renal toxic effect was observed in monkeys given high doses of triazinate (20 and 40 mg/kg/day or 240–480 mg/m²/day) for 5 days.

[Cancer Chemother Rep 59:523–530, 1975]

Triazinate (fig 1), a reversible inhibitor of dihydrofolate reductase, has shown antineoplastic activity against both the ascitic and

[1] Received Nov 19, 1974; revised Feb 4, 1975; accepted Feb 19, 1975.

[2] Supported by contracts NIH-70-2089 and NO1-CM-33711 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Triazinate: CAS reg. No. 41191-04-2; ethanesulfonic acid, compd. with α-[2-chloro-4-(4,6-diamino-2,2-dimethyl-*s*-triazin-1(2*H*)-yl)phenoxy]-*N*,*N*-dimethyl-*m*-toluamide (1:1); TZT. Supplied by Drug Research and Development, DCT, NCI.

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[5] The authors would like to acknowledge the excellent technical and administrative assistance of Patricia Stewart, Christopher DeNeen, and Irene Mark.

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intramuscular forms of Walker 256 carcinosarcoma and the Dunning ascitic leukemia (1) but has been inactive against the L1210 tumor, probably due to the relatively lower accumulation of intracellular drug levels (2). This compound has a high level of aqueous solubility which should facilitate clinical usage (3). A second desirable property of this agent is the relative ease with which it transverses the blood-brain barrier in dogs (4). This could offer a therapeutic advantage over methotrexate [7] which must be administered by intrathecal injection to effectively treat meningeal leukemia, occasionally resulting in severe toxicity (5).

This report will describe preclinical toxicology trials conducted in registered beagle dogs and rhesus monkeys, with the drug administered intravenously (iv) at various dose regimens. The correlation between the in vitro sensitivity of bone marrow to triazinate and toxic effects will also be presented.

MATERIALS AND METHODS

Triazinate was supplied as bulk powder. It

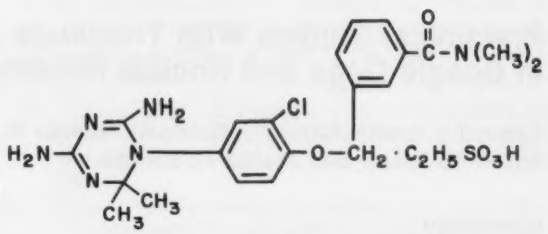

FIGURE 1.—Triazinate structure.

was weighed daily and 1% aqueous solutions were prepared and bacteriologically sterilized by being passed through a 0.22μ filter (Millipore Corporation, Bedford, Mass). All doses (table 1) were determined in milligrams based on each animal's weight in kilograms [8] and administered at a rate of 10 mg/kg/minute.

The protocols used for these studies were designed following the principles outlined by Prieur et al (6).

Animal Studies

Registered beagles, 5½–12 months of age and weighing 7–10 kg, and conditioned *Macaca mulatta* (rhesus) monkeys, ages unknown and weighing 3–5 kg, were purchased commercially and housed in stainless-steel cages within temperature-humidity controlled rooms. Food and water were available ad libitum but were

[7] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-; MTX.

[8] Conversions to drug concentration/body surface (m^2) which appear in this report were calculated by using the formula of Freireich et al (7)—body weight (kg) × km factor = m^2.

TABLE 1.—Distribution by dose and schedule of dogs and monkeys receiving iv triazinate*

	Dose levels		No. of animals treated with—		
Species	Mg/kg/day	Mg/m^2/day	Single dose	5 Daily doses	Weekly dose, 6 wks
Dog	40	800	2		
	20	400	2		
	10	200	2		2
	5	100	2	2	2
	2.5	50		4	2
	1.25	25		2	
	0.625	12.5		2	
	0.3125	6.25		2	
Monkey	40	480		2	
	20	240		2	
	10	120		2	
	5	60		2	
	2.5	30		2	

*Each group included equal numbers of male and female animals.

withheld 12 hours before blood and urine samples were taken. Immediately upon arrival, all animals were weighed and checked for physical defects. Monkeys were conditioned for 4 weeks after arrival. The conditioning included an initial chest radiograph and three tuberculin tests at 2-week intervals.

Blood was obtained for baseline determinations twice before dosing was initiated and at periodic intervals during the study. Blood analyses included hematocrit; hemoglobin; red blood cell (rbc), white blood cell (wbc), differential, platelet, and reticulocyte counts; and serum alkaline phosphatase, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, total and direct bilirubin, prothrombin time, blood urea nitrogen (BUN), glucose, sodium, potassium, chloride, magnesium, and calcium.

In addition, complete physical examinations included body weight, body temperature, and electrocardiographic and ophthalmoscopic examinations. At the end of each treatment period, one animal was selected from each group and killed by exsanguination after anesthesia with pentobarbital sodium. All organs were examined grossly and tissue specimens were collected for microscopic examination. The second animal from each group was retained for 45 days without treatment and observed in the same manner for delayed toxicity or remission of toxic effects.

In Vitro Studies

The pharmacologic effect of triazinate on DNA synthesis by isolated bone marrow cells from a separate group of three dogs and three monkeys was studied by the following procedure. Ten ml of heparinized (20 units/ml) bone marrow was collected from the proximal humerus with the animals under light surgical anesthesia induced by iv pentobarbital sodium. Each sample was allowed to settle with the collecting tubes at a 45° angle. The dog samples were first mixed with equal parts of homologous serum to facilitate sedimentation. After 1 hour, the leukocyte-rich plasma was collected separately from each tube and adjusted to a concentration of 10–30 $\times$ 10^6 cells/mm^3 with Eagle's minimal essential medium containing 10% horse serum. A sample of each cell suspension was prepared and examined microscopically to ensure a uniformity of cell types among animals and between species.

The effect of triazinate on cellular dihydrofolate reductase activity was analyzed by using the procedure described in detail by Capizzi (8). Separate aliquots of each cell suspension were treated in the same manner with equal concentrations of methotrexate as a positive control. In summary, rates of DNA syntheses were measured by the time-dependent incorporation of tritiated deoxyuridine (^{3}H-UdR) from the suspending medium. Aliquots of cells were analyzed for radioactivity at 10-minute intervals and the uptake (dpm/10^6 cells/minute of incubation) was calculated by linear regression analyses. Final results are expressed as a percentage of the saline-treated control incorporation.

RESULTS

Only clinical or pathologic alterations considered to be direct toxic effects and unequivocally drug related are described.

Mortality

Five daily doses of 2.5 mg/kg/day (50 mg/m^2/day) of triazinate caused mortality in dogs, while doses of 5 mg/kg/day (60 mg/m^2/day) were nontoxic in monkeys. A level of 40 mg/kg (480 mg/m^2) per day or 16 times the lethal canine level was required to produce mortality in the rhesus monkey.

Clinical Signs of Acute Toxicity

An acute, generalized response occurred after iv injections of this compound to animals of both species at doses greater than 5 mg/kg. This consisted of ataxia and dyspnea, followed by coma and death, apparently from respiratory arrest since heart action continued after complete apnea. Slow administration of daily injections of this agent to monkeys at high dose levels permitted an accurate characterization of this effect from onset to termination. The earliest signs of impending toxicity appeared randomly without any specific dose relationship and were manifested as a piloerection and a

pronounced shift from thoracic to abdominal breathing. Continued infusion led to cyanosis, restlessness, and signs of a generalized muscle weakness. The cardiovascular reflexes showed no adverse effects since the heart rate usually increased with a strong steady pulse. Respiratory depression developed as a terminal event. A normal or elevated heart rate with normal electrocardiographic patterns continued if respiration was maintained by alternating thoracic compression. No clinical indications of cholinergic stimulation were detectable such as meiosis or bradycardia. The postinjection effect was brief; all injected monkeys appeared completely recovered from within 5 to 10 minutes and would commonly accept and eat fruit within 30 minutes.

Gastrointestinal Toxicity

In both dogs and monkeys, toxic doses caused signs of gastrointestinal toxicity, but qualitative and quantitative differences between the two species were evident.

In dogs, a fatal dose-related enteritis appeared 3–5 days after the onset of daily drug administration which ranged from soft feces at 0.625 and 1.25 mg/kg/day (12.5 and 25 mg/m²/day) to profuse hemorrhaging at 2.5 and 5.0 mg/kg/day (50 and 100 mg/m²/day). The latter was accompanied by hemoconcentration, ie, elevated hematocrit, hemoglobin, and rbc counts, and depressed serum chloride values (table 2). At necropsy, duodenal and jejunal mucosae were covered by a thick tenacious bile-stained material. Microscopically, the tips of the villi were denuded of epithelium and covered by an eosinophilic staining material containing numerous colonies of bacteria. Submucosal capillaries and small venules were moderately dilated by fresh blood. Similar alterations continued into the colon where the superficial mucosa showed generalized depletion of the epithelial lining. All the deep mucosal glands were dilated and contained flattened epithelium and a few desquamated cells. In small focal areas, the lamina propria was infiltrated by neutrophils and, as in the small intestine, considerable vascular dilatation was present. The esophageal mucosa was lined by a thin pseudomembrane, consisting of fibrin with entrapped neutrophils and desquamated epithelial cells. Otherwise, no microscopic evidence of cellular degeneration was observed in this organ.

A different gastrointestinal response appeared in monkeys that were treated by the same drug regimen as the dogs. A mild diarrhea, without melena, occurred only at the highest dose levels, 20 and 40 mg/kg/day (240 and 480 mg/m²/day), administered for 5 days. Neither dehydration nor hemoconcentration occurred; serum chloride levels were stable (table 3). At the highest drug level (40 mg/kg/day), minimal lesions occurred which were confined to the colon comprising inflammatory cell infiltration and focal-to-diffuse villar cellular desquamation. Mild intestinal changes, dilated glands, and enlarged lining cells in the crypts of the large

TABLE 2.—Clinical responses relating to adverse gastrointestinal effects of triazinate given iv for 5 daily doses

Species	Animal No./sex	Dose		Time (day)	Hemoconcentration			Serum chloride (mEq/liter)	Degree of diarrhea
		Mg/kg/day	Mg/m²/day		Hct (%)	Hgb (g/100 ml)	Rbc (× 10⁶ cells/mm³)		
Rhesus monkey	314/F	20	240	0	40	12.5	5.8	114	
				6	34	10.5	4.9	108	Mild
	432/M	20	240	0	40	13.5	6.2	114	
				6	40	13.0	5.4	108	Mild
Beagle dog	2/F	2.5	50	0	44	16.0	6.5	106	Profuse,
				6	63	23.5	10.6	75	hemorrhagic
	1/M	2.5	50	0	47	16.0	7.0	114	Profuse,
				6	50	18.0	7.5	93	hemorrhagic

TABLE 3.—Effect of triazinate on the rate of ^{3}H-UdR incorporation into DNA by isolated bone marrow cells from beagle dogs and rhesus monkeys

Drug concentration (M)	Rate of ^{3}H-UdR incorporation as % of control values	
	Dogs*	Monkeys*
5×10^{-7}	80	90
2×10^{-6}	10	23†
1×10^{-5}	1	1

*Figures represent values for 3 animals.
†Significantly less than dogs by "student's t" test ($P < 0.01$).

and small intestines were produced by 20 mg/kg/day.

Gastrointestinal toxicity was a reversible effect in animals of either species which survived treatment. Clinical signs disappeared within 72 hours in animals retained for observation after treatment and no permanent changes were discovered at the necropsy which followed 6 weeks without treatment.

Hematopoietic Toxicity

Dog bone marrow function was depressed by dose levels of 2.5 mg/kg (50 mg/m^2) given for 5 days. This was demonstrated by a circulating neutropenia within 48 hours (fig 2), plus lesions which were found at necropsy. Grossly, hyperchromic marrow appeared in the long bones from affected animals. Microscopically, precursor cells in both the myeloid and erythroid series were depleted, with vacant areas replaced by prominent, blood-filled sinusoids. In monkeys, signs of bone marrow dysfunction were absent despite dose levels almost five times higher than in dogs (table 2). Blood neutrophils were stable or elevated (fig 3); at necropsy, an occasional megaloblastoid cell was the only evidence that any toxic response had occurred in this organ at the lethal dose level of 20 mg/kg/day for 5 days. All other marrow cells appeared to be histologically normal.

The results of in vitro drug on the isolated bone marrow cells parallel these clinical findings. Triazinate concentrations of 10^{-7} M had little pharmacologic effect, while 10^{-5} M prevented uptake of the DNA precursor, ^{3}H-UdR, in both species (table 3). A significant differential effect was observed with drug concentrations of 10^{-6} M. DNA synthesis in triazinate-treated bone marrow cells from monkeys was more active compared with a preparation of cells from dog marrow cells (table 3) which were treated in the same manner.

Lymphoid tissue was uniformly depleted of cells in both species, although monkeys required almost a fivefold higher dose to achieve the same effect. A reversible lymphopenia (figs 2 and 3) correlated with drug-damaged germinal centers in all lymphoid tissues of animals necropsied 24 hours after the final treatment.

Renal Toxicity

Several indications of kidney dysfunction, ie, elevated BUN and creatinine levels, appeared in animals of both species that received the highest dose levels. No drug-induced lesions at the microscopic level were detectable in the dogs after necropsy. In a monkey that received 40 mg/kg/day for 5 days and died 5 days later, clinical signs of nephrotoxicity were accompanied by hyperkalemia, ie, serum level of 11 mEq/liter. Histologically, the proximal tubules had lost the epithelial lining cells and contained proteinaceous casts.

Other Changes

When the dose regimen was changed from a single or five daily consecutive doses to the extended intermittent schedules, a reversible alopecia appeared in one dog after 5 weeks of treatment with single doses of 10 mg/kg/week. Otherwise, this schedule did not alter the toxicity pattern which has been described.

DISCUSSION

It might be expected that a drug such as triazinate which affects the biosynthesis of a basic nuclear material would have similar toxic effects in a variety of animal species. Many cytotoxic agents have the common toxic property of adversely affecting those organ systems which require large numbers of rapidly dividing cells for proper functioning, such as bone marrow tissue and the gastrointestinal epithelium (9). This study shows that at least one drug which inhibits DNA synthesis can have divergent toxic effects in two species with different mechanisms of drug-induced lethality.

The dog mortality is believed to follow a

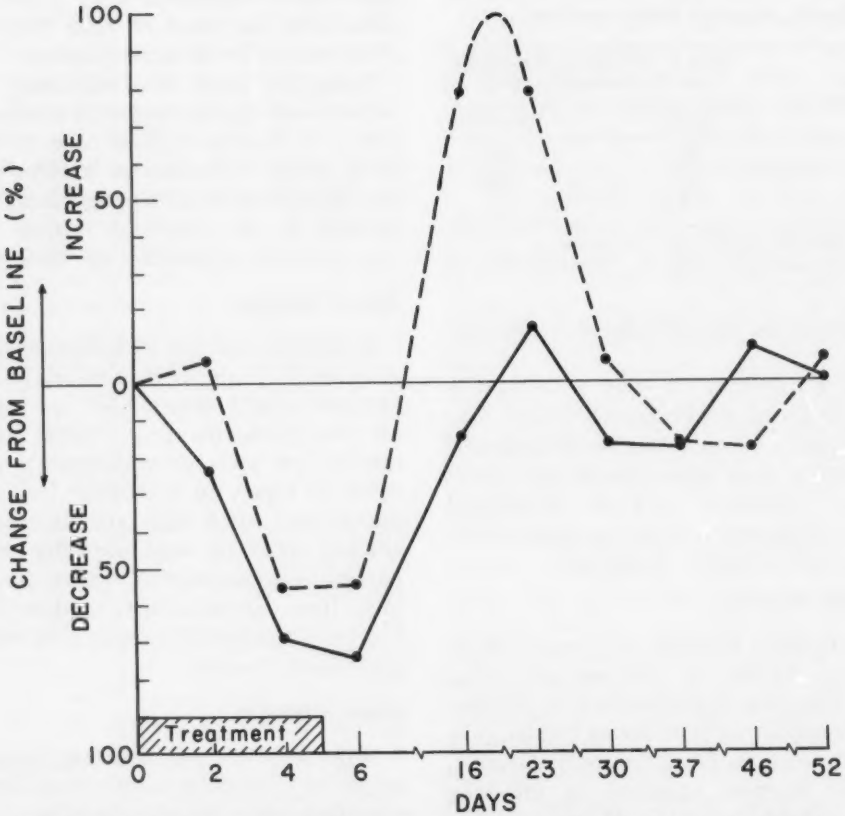

FIGURE 2.—Response of circulating leukocytes to 5 daily triazinate injections (2.5 mg/kg/day iv) in the beagle dog. ——— = Lymphocytes and - - - = neutrophils.

toxic destruction of the gastrointestinal tract leading to a fatal dehydration and shock. The monkeys, on the other hand, had little difficulty tolerating a milder form of this toxicity. This relatively resistant gastrointestinal tract made it possible to reach drug dose levels in monkeys 9.5 times the lethal dog level, or 50 vs 480 mg/m²/day for 5 days, respectively. In monkeys, the fatal lesion appeared to be hyperkalemia after severe renal toxicity. Presumably, the dogs would have shown similar kidney damage if the same high drug dose levels were attained. The dog, in fact, did show clinical signs of renal dysfunction, but only those animals which were dehydrated by severe diarrhea and adipsia. In this case, uremia appeared to be a pre-renal phenomenon since correlating histopathologic renal lesions were absent at necropsy.

The rhesus kidney damage is consistent with a report of renal toxicity in patients receiving methotrexate (10). A similar effect in animals has not been previously reported with this class of anticancer agents.

While bone marrow toxicity might have been expected in both species, it appeared only in the dog. Monkey bone marrow was relatively more drug resistant both in vitro and in vivo. As illustrated in figure 3, hematologic function in the rhesus monkey was unchanged, and the bone marrow appeared to be capable of producing a neutrophilia even though the animal received near lethal doses of this drug. The megaloblastosis, which was observed in both monkeys which received the highest dose level, indicates that the marrow is exposed to this drug and is not completely insensitive. The

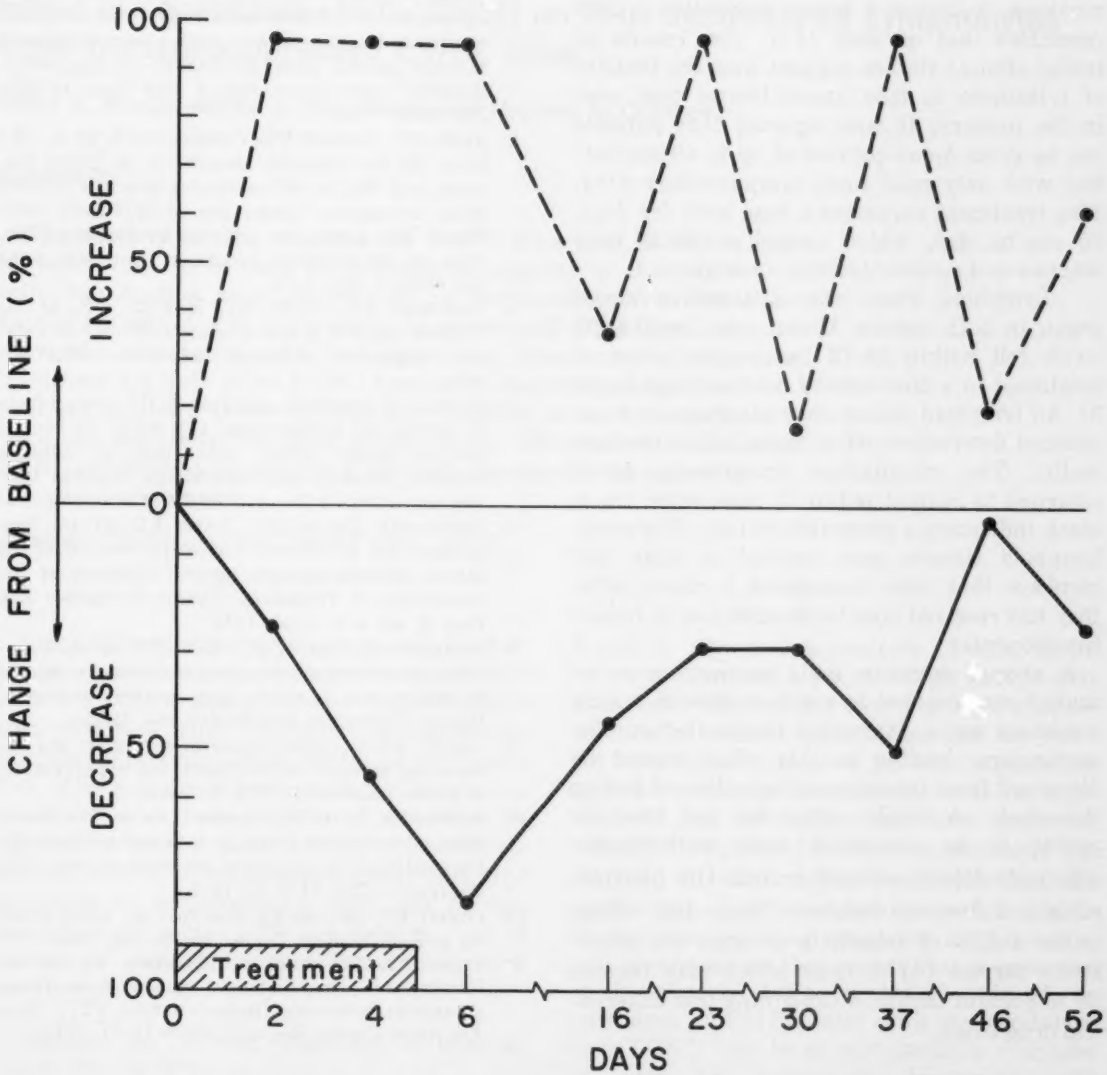

FIGURE 3.—Response of circulating leukocytes to 5 daily triazinate injections (20 mg/kg/day iv) in the rhesus monkey. ——— = Lymphocytes and - - - = neutrophils.

results from our study on drug effects in isolated bone marrow cells show that a differential sensitivity can be demonstrated in vitro at the cellular level. While high concentrations of triazinate (10^{-5} M) inhibited ^{3}H-UdR uptake in marrow cells from both species and low concentrations (10^{-7} M) had little effect, an intermediate concentration (10^{-6} M) significantly inhibited the cellular uptake of this labeled precursor in dog marrow cells compared with those from the monkey (table 3).

The available evidence suggests that these two species' differences with regard to the toxicity of triazinate are probably unrelated to a different rate of metabolism and excretion. Cashmore et al (4) examined the serum half-

life of triazinate in a variety of laboratory animals and found it to be similar in dogs and monkeys, 3–4 vs 2–4 hours, respectively; this resembles that of man (11). The results of initial clinical studies suggest that the toxicity of triazinate in man approximates that seen in the monkey. It was reported that patients can be given 5-day courses of up to 40 mg/m²/day with only mild toxic symptomology (11). This treatment surpasses a dose level for dogs, 25 mg/m²/day, which caused nonfatal bone marrow and gastrointestinal toxic effects.

Lymphoid tissue was a sensitive target organ in both species. Circulating lymphocyte levels fell within 48–72 hours after onset of treatment in a dose-related manner (figs 2 and 3). All lymphoid tissues showed extensive drug-induced destruction when examined microscopically. The circulating lymphocyte levels returned to normal within 1 week after treatment, indicating a reversible toxicity. Moreover, lymphoid tissues were normal in dogs and monkeys that were necropsied 6 weeks after they had received dose levels sufficient to induce lymphopenia.

A second common toxic property was an acute toxic response in which a skeletal muscle weakness was a prominent manifestation. The mechanisms leading to this effect cannot be discerned from the information collected during this study. A similar effect has not been reported to be associated with methotrexate administration in animals or man. One pharmacologic difference between these two drugs is the ability of triazinate to cross the blood-brain barrier (4) in dogs which may become an important factor in explaining this differential drug effect.

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Effect of Levamisole (NSC-177023) on DNA Synthesis by Lymphocytes From Immunosuppressed C57BL Mice [1,2]

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SUMMARY

The effect of in vivo treatment of C57BL mice with BCNU and/or levamisole on the in vitro DNA synthetic capacity of their spleen cells was studied as a measure of cell-mediated immune function. BCNU treatment was suppressive to spleen cell DNA synthesis; conversely, treatment with levamisole was stimulatory. Levamisole treatment 5–8 days after BCNU treatment resulted in significant recovery of DNA synthetic capacity. Multiple doses of levamisole were not more effective than single doses. Allogeneic stimulation of BCNU-suppressed lymphocytes was not consistently increased by levamisole treatment.

[Cancer Chemother Rep 59:531–536, 1975]

Since cytoreductive agents currently used in cancer chemotherapy are directed toward cells in active DNA synthesis, one of the undesirable side effects of such treatment is nonspecific immunosuppression due to inhibition of replication of cells in the immune system. This results in depletion of cells capable of mounting an adequate defense against both infection and recurrent growth of residual malignant cells. One approach to counteracting such loss of immunologically active cells has been the use of chemically defined immune stimulators, such as tilorone (1–3),[5] poly-I:poly-C (4,5), poly-A:poly-U (6), and levamisole (7–12), to increase the immune capacity of the host shortly after cytoreductive therapy. The active target reaction of such stimulatory agents has often not been defined; however, in vitro stimulation of DNA synthesis of lymphoid cells by poly-I:poly-C and levamisole has been described (13–16). The present series of experiments was designed to explore the possibility that the mechanism of action of levamisole is indeed to act via stimulation of depressed DNA lymphocyte synthesis; we thus report that suppression of lymphocyte DNA synthesis induced by treatment of C57BL mice with the antitumor drug BCNU [6] can be at least partially overcome in vivo by subsequent treatment with levamisole.

MATERIALS AND METHODS

The studies were carried out in normal

[1] Received Dec 17, 1974; revised Feb 18, 1975; accepted Feb 19, 1975.

[2] Levamisole: CAS reg. No. 16595-80-5; imidazo[2,1-*b*]thiazole, 2,3,5,6-tetrahydro-6-phenyl-, monohydrochloride. Supplied by Janssen Pharmaceutica, Beerse, Belgium.

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[4] *Reprint requests to:* Dr. Wilna A. Woods, VB, VO, DCCP, NCI, Bldg 37, Rm 1D14, Bethesda, Md 20014.

[5] Hoffman PF, Ritter HW, and Krueger RR. Tilorone hydrochloride stimulation of the primary immune response of mice. Presented at the 7th International Congress of Chemotherapy, Prague, 1971.

Tilorone; NSC-143969; CAS reg. No. 27591-69-1; fluoren-9-one, 2,7-bis[2-diethylamino)ethoxy]-, dihydrochloride.

[6] BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, NCI.

C57BL/6 male mice, 6–8 weeks of age. Allogeneic target cells for mixed lymphocyte cultures were obtained from 6–8-week-old male DBA/2 mice.

Experimental animals were treated intraperitoneally (ip) on Day 0 with 30 mg/kg of BCNU. This represents the standard tumor remission-inducing dose employed in our laboratory (12). They were then treated with levamisole in a volume of 0.2 ml given ip in normal saline at various times after BCNU treatment. Controls included untreated animals as well as animals treated with either BCNU or levamisole alone.

Five C57BL mice were killed at various intervals after levamisole treatment and pooled spleen cell suspensions were prepared for effector cells in RPMI-1640 medium with 20% fetal bovine serum (RPMI-20) according to published procedures (16). DBA/2 target cell suspensions were prepared by treating spleen cell suspensions with 75 μg/ml of mitomycin C [†] at 37° C for 30 minutes. Preparation and labeling of cultures for DNA synthetic determinations of both stimulated and nonstimulated lymphocytes have been described (16). Briefly, DNA synthesis by nonstimulated lymphocytes was determined by culturing 1×10^6 nucleated spleen cells for 72 hours in RPMI-20 and pulsing for 5 hours with 1 microcurie of ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass; specific activity, 200 millicuries/mmol).

One-way mixed lymphocyte cultures were initiated with 3×10^6 nucleated cells/2 ml of RPMI-20 at a target-cell:effector-cell ratio of 2:1 and were similarly pulsed with ^{3}H-thymidine.

^{3}H-thymidine-labeled cells were collected on glass fiber millipore filters, and the DNA was precipitated with cold 10% trichloroacetic acid. Individual filters were placed in scintillation vials and dried; 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass) was added, and acid-precipitable counts were determined by liquid scintillation spectrometry with a Packard TriCarb Liquid Scintillation Counter. Statistical significance of differences of the means of the various treatment groups was determined by the "Student's t" test.

[†] Mitomycin C: NSC-26980; CAS reg. No. 50-07-7.

RESULTS

Effect of BCNU and Levamisole on DNA Synthesis of Spleen Cell Cultures

We attempted to overcome depression of spleen cell DNA synthesis due to BCNU treatment by subsequently treating the mice with levamisole. The results of typical experiments are presented in figure 1 and table 1. In the experiment shown in figure 1, C57BL/6 mice were treated on Day 0 with 30 mg/kg of BCNU, and were given 10 mg/kg of levamisole on Day −1, 0, +1, +7, +14, or +21. Five mice from each combined treatment group, as well as mice treated with BCNU or levamisole alone, were killed at the intervals indicated in figure 1. Spleen cell cultures were prepared, pulsed with ^{3}H-thymidine after 72 hours, and assayed for DNA synthesis.

The DNA synthetic capacity of spleen cell cultures prepared from C57BL mice treated with BCNU was decreased on the first, third, and tenth day after treatment. This depression was not seen on the seventh day and such transient recovery was regularly observed from 7 to 10 days after treatment (fig 1, top panel). Thereafter, DNA synthesis was depressed and did not return to normal levels for at least 20 days after treatment.

In contrast, a marked enhancement of DNA synthesis was noted in spleen cell cultures (fig 1, top panel) prepared from mice treated with 10 mg/kg of levamisole. The enhancement was not seen after 10 days.

The middle panel of figure 1 shows the lack of effect of levamisole given 1 day before or at the same time as BCNU. The bottom panel shows that when given 1 day after BCNU treatment or thereafter, DNA synthesis of effector cells had returned to normal values within 5–8 days of levamisole treatment.

Table 1 presents the results of another experiment to determine the optimum time for levamisole treatment after BCNU suppression. The data are reported normalized to the amount of ^{3}H-thymidine incorporated by cells from animals treated with BCNU only, so that a

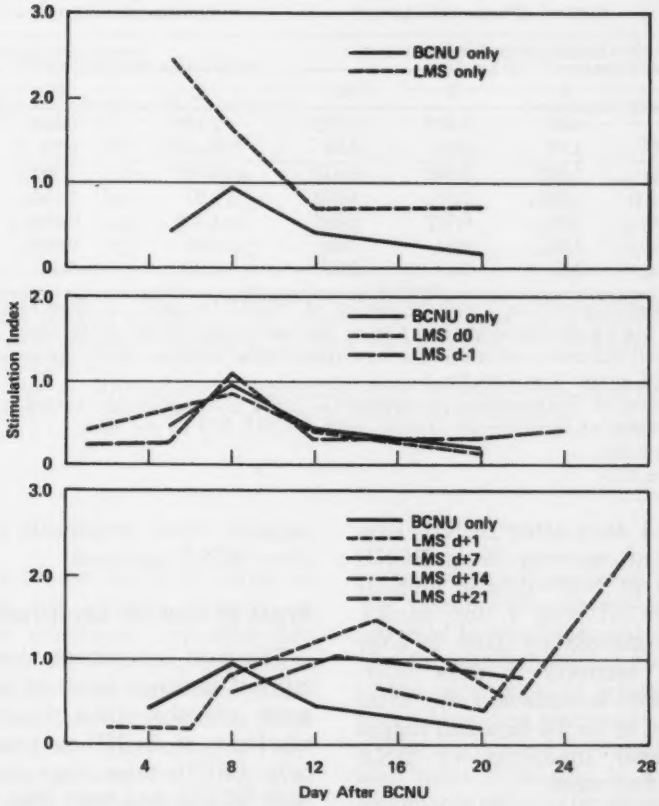

FIGURE 1.—Effect of levamisole on recovery of DNA synthetic capacity of spleen cells derived from BCNU-treated C57BL mice. C57BL mice were treated ip on Day 0 with 30 mg/kg of BCNU; 10 mg/kg of levamisole was given on Day −1, 0, +1, +7, +14, or +21. Spleens were removed and cell suspensions were prepared containing 1×10^6 cells in 2 ml of RPMI-20. The cultures were incubated at 37°C for 72 hrs and pulsed with 1 microcurie of ^{3}H-thymidine for 5 hrs. The cells were collected on glass fiber filters, and the DNA was precipitated with cold 10% trichloroacetic acid. The filters were dried and placed in 10 ml of Aquasol, and counts were determined by liquid scintillation spectrophotometry. Results are reported as stimulation index:

$$\frac{\text{dpm in cultures from treated animals}}{\text{dpm in cultures from control untreated animals}}.$$

ratio of > 1.0 indicates recovery of synthetic capacity over the depression caused by the BCNU treatment, and a ratio of < 1.0 indicates depression above that due to BCNU. BCNU was given on Day 0 and levamisole was given on Day 1, 5, 8, or 14. Animals were killed on Days 1 and 4 after levamisole treatment, and then every 7 days after treatment. The data are presented so that the time of recovery from BCNU treatment can be determined in relation to the levamisole treatment.

Treatment with levamisole resulted in significant recovery of lymphocyte DNA synthesis (table 1, left) within 1 day of treatment when

TABLE 1.—Effect of levamisole on DNA synthesis of BCNU-suppressed C57BL lymphocytes*

Day animals were killed after levamisole treatment	Control effector cells alone†				Allogeneic stimulated effector cells (mitomycin C-treated DBA target cells)†			
	Levamisole given on Day—				Levamisole given on Day—			
	1	5	8	14	1	5	8	14
1	0.90	0.88	1.45¶	2.53‡	2.16‡	0.83‡	1.07	1.06
4	0.58‡	1.01	1.22	0.95	0.49‡	0.85	1.04	1.00
8	0.63§	1.83‡	2.34‡	0.71‡	0.76**	1.30‡	1.18	1.26
14	1.51§	1.37‡	1.08	1.85‡	0.87	1.45‡	1.29‡	0.85‡
21	0.73¶	1.16	0.72‡	1.39‡	1.30‡	0.93¶	0.75‡	0.55‡
28	1.41‡	1.09	ND	ND	0.80	0.85¶	ND	ND
35	1.50‡	ND	ND	ND	0.99	ND	ND	ND

*C57BL mice were treated ip on Day 0 with 30 mg/kg of BCNU. On Day 1, 5, 8, or 14 separate groups of mice were treated ip with 10 mg/kg of levamisole and 5 mice per group were killed at the times indicated after levamisole treatment. Spleen cell cultures and one-way mixed lymphocyte cultures were prepared and DNA synthesis was assayed as described in the legend to fig 1.

†Figures represent ratio of ^{3}H-thymidine incorporation (dpm) of lymphocytes treated with BCNU and levamisole to similar incorporation of lymphocytes treated with BCNU. ND = not done.

‡$P < 0.005$. ¶$P = 0.025$.

§$P = 0.01$. **$P = 0.05$.

administered 8 or 14 days after BCNU, augmenting the transient recovery from BCNU which was observed at 7–10 days in animals treated only with BCNU (fig 1, top panel). Treatment with levamisole on Day 5 after BCNU resulted in recovery 8 days later, whereas treatment with levamisole 1 day after BCNU was not effective for 14 days and indeed resulted in additional inhibition of DNA synthesis during the first week.

Effect of BCNU and Levamisole on Allogeneic Stimulation of DNA Synthesis

Enhancement of DNA synthesis of one-way mixed lymphocyte cultures was not regularly observed, indicating that levamisole is not capable of further stimulating cells which have already responded to allogeneic stimulation. The effect of levamisole on allogeneic stimulation of DNA synthesis (table 1, right) was most effective when given on Day 5 after BCNU treatment. Although treatment on the first day after BCNU resulted in an early stimulation on the next day, a significant depression of DNA synthetic capacity was seen for 14 days thereafter. These results again point to (*a*) activity of levamisole in suppressed animals, (*b*) frequent inability of levamisole to augment stimulation due to allogeneic interaction, and (*c*) significant depression of DNA synthetic capacity when levamisole is given very early after BCNU treatment.

Effect of Multiple Levamisole Treatments

The next experiment was designed to determine if multiple doses of levamisole would be more effective than single doses on DNA synthesis of BCNU-suppressed C57BL spleen cells. C57BL mice were treated ip on Day 0 with BCNU, and were then given a single dose (Day 1, 8, or 15) or multiple doses (Days 1 and 8, or Days 1, 8, and 15) of 10 mg/kg of levamisole. Animals from each group were killed on Day 1, 4, 8, or 15 after levamisole treatment. Results are again given to show enhancement (values > 1.0) or depression (values < 1.0) of ^{3}H-thymidine incorporation by spleen cells from animals given BCNU and levamisole in comparison to the level in cultures derived from animals given BCNU alone (table 2). Stimulation of DNA synthesis of spleen cells with multiple treatment was not more than with single treatment, and indeed DNA synthesis due to allogeneic stimulation was depressed by multiple treatment.

DISCUSSION

In these experiments, we have attempted to determine the mechanism of in vivo immune stimulatory action of levamisole reported pre-

TABLE 2.—Effect of multiple doses of levamisole on DNA synthesis of BCNU-suppressed C57BL lymphocytes*

Day animals were killed after last levamisole dose	Control effector cells alone†					Allogeneic stimulated effector cells (mitomycin C-treated DBA target cells)†				
	Levamisole given on Day(s)—					Levamisole given on Day(s)—				
	1	8	15	1,8	1,8,15	1	8	15	1,8	1,8,15
1	0.14‡	0.93	1.20‡	1.38	0.93	1.12	1.38¶	0.70§	0.86‡	0.81‡
4	1.24	1.20	ND	1.81‡	ND	0.63‡	1.12‡	ND	0.74‡	ND
8	1.01	0.94	0.99	0.92	0.82§	1.02	0.57‡	0.78§	0.59‡	0.60‡
15	1.08**	1.11**	ND	0.56‡	ND	0.49‡	0.44‡	ND	0.44‡	ND

*C57BL mice were treated ip on Day 0 with 30 mg/kg of BCNU. Animals were treated with single doses (Day 1, 8, or 15), with 2 doses (Days 1 and 8), or with 3 doses (Days 1, 8, and 15) of levamisole. Animals were killed on the days indicated after levamisole treatment. Cultures were prepared and DNA synthesis was assayed as described in the legend to figure 1.

†Figures represent ratio of ^{3}H-thymidine incorporation (dpm) of lymphocytes treated with BCNU and levamisole to similar incorporation of lymphocytes treated with BCNU. ND = not done.

‡$P < 0.005$.

§$P = 0.01$.

¶$P = 0.025$.

**$P = 0.05$.

viously (12) and to follow the time course in order to determine the optimal time(s) of treatment. We have previously reported that levamisole is mitogenic for lymphocytes in vitro (5, 16), primarily under conditions suboptimal for DNA synthesis, but does not stimulate DNA synthesis by fibroblasts and macrophages (16). The same stimulatory activity for DNA synthesis of lymphoid cells, taking place in vivo, would permit an earlier return to immune competence to a variety of antigens by nonspecifically replenishing the lymphoid population after cytoreductive therapy. The accelerated recovery of lymphoid germinal centers in mice treated with BCNU and levamisole reported by Perk et al (17) would thus be explained by early stimulation of lymphoid cell proliferation. The experiments reported here support such a hypothesis, since treatment with levamisole resulted in increased DNA synthetic capacity of unstimulated lymphocytes but was not consistently effective in increasing that of lymphocytes already undergoing allogeneic stimulation by mixed lymphocyte culture reactions. Furthermore, levamisole manifested its stimulatory effect most dramatically when the lymphocyte DNA synthetic capacity was inhibited by BCNU treatment, and it only transiently stimulated DNA synthesis above normal values when administered without suppression. The immune stimulatory activity of levamisole reported by our laboratory (12) and by others (7–11) would therefore be due to replenishing of the population of lymphocytes available for specific immune reactions.

The augmented depression of DNA synthesis seen most strikingly in animals treated with levamisole early after drug suppression is possibly due to the more effective activity of the BCNU against the additional cells placed in S phase of the cell cycle by the levamisole treatment. The ultimate mechanism for stimulation has been suggested by the effect of levamisole on the cyclic nucleotide levels via interaction with the cyclic nucleotide phosphodiesterases (16,18). Investigation of the specific activity of these enzymes in lymphoid cells during therapy as well as the availability of the enzymes at the cell membrane during different phases of the cell cycle may also lead to an explanation of the occasional depressive activity of the drug.

From the data presented, the optimum time for treatment with levamisole appears to be 5–8 days after BCNU therapy. Indeed, the most effective time of treating with levamisole in a combined modality treatment of a murine leukemia was shown to be effective 6 or 10 days after BCNU treatment (12). It remains to be

determined if additional treatments with levamisole at time intervals different than those tested in these experiments will enhance the stimulation seen with single doses.

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Remission Maintenance of Acute Nonlymphoblastic Leukemia With BCNU (NSC-409962) and Cyclophosphamide (NSC-26271) [1,2,3]

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SUMMARY

Thirteen of 29 patients with acute nonlymphoblastic leukemia achieved complete remission with cyclophosphamide, cytosine arabinoside, and vincristine, and remissions were maintained with a combination of BCNU and cyclophosphamide. The maintenance drugs (200 and 1000 mg/m^2 respectively) were given at 8-week intervals intravenously. Only six of the 13 patients achieving a complete remission have relapsed. The projected median duration of complete remission is 65 weeks and of survival from diagnosis is 144 weeks. These remission and survival durations compare favorably with the results achieved using other forms of remission-maintenance therapy. The advantage of our form of maintenance therapy is that only overnight hospitalization is required at 8-week intervals.

[Cancer Chemother Rep 59:537–545, 1975]

A variety of drug combinations which include cytosine arabinoside and/or daunorubicin induce remission in 40%–50% of adult patients with previously untreated acute nonlymphoblastic leukemia (1). If treatment is not continued these remissions are usually short; a median duration of 5 weeks was reported when cytosine arabinoside was utilized alone for remission induction (2). Maintenance therapy is therefore important. We wish to report on the use of a combination of BCNU and cyclophosphamide as an effective means of prolonging remission in these patients.

MATERIAL AND METHODS

Patient Selection and Diagnostic Criteria

Forty adults (all older than 15 years) with previously untreated acute nonlymphoblastic leukemia were admitted to the Department of Medicine of the Princess Margaret Hospital between July 1971 and December 1973. Eleven of these patients were not entered into this study either because of extramedical reasons (one patient), advanced age (two patients), or apparent smoldering disease (seven patients); one patient died immediately after admission to the hospital before remission-induction therapy could be started. The remaining 29 patients (17 women and 12 men) ranged in age from 16 to 81 years with a median of 41 years. Sixteen had

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[2] Supported by grants from the Ontario Cancer Research and Treatment Foundation (No. 236), the Medical Research Council (MT-1420), and the National Cancer Institute of Canada (No. 3106).

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. BCNU was supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

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[5] *Reprint requests to:* Dr. J. E. Curtis, Department of Medicine, Ontario Cancer Institute, 500 Sherbourne St, Toronto M4X 1K9, Ontario, Canada.

acute myeloblastic leukemia, five had acute promyelocytic leukemia, two had erythroleukemia, and six had acute myelomonocytic leukemia.

Diagnosis was made from the appearance of Wright-stained peripheral blood and bone marrow films, using standard criteria previously described (3). Serum muramidase levels [*] were measured at the time of diagnosis in most of the patients; values were either normal or elevated prior to treatment (4).

Remission-Induction and Consolidation Treatment

The remission-induction protocol has been described previously (6). In brief, treatment consisted of repeated 4-day courses of cyclophosphamide and cytosine arabinoside, each drug given at 40 mg/m² of body surface area every 8 hours for a total of 12 doses, with a single injection of 2 mg of vincristine on Day 1 for each course (CAV protocol). These courses of CAV were repeated at 14–21-day intervals until remission. Criteria for complete remission (CR) were less than 5% blast cells and normal hemopoiesis in the bone marrow, more than 1500 granulocytes/mm³ and 100,000 platelets/mm³ in the blood, and no clinically detectable disease.

Consolidation, after remission induction, consisted of repetitive courses of CAV every 14–21 days until three successive bone marrow aspirates met the criteria for CR. At least two CAV courses were therefore necessary for consolidation, and usually more were required.

Maintenance Treatment

After the last course of consolidation therapy patients received no treatment for 3–4 weeks, at which time, if the CR was sustained, maintenance therapy with BCNU and cyclophosphamide was started. BCNU (200 mg/m²) and cyclophosphamide (1000 mg/m²) were given in single intravenous "push" injections at 8-week intervals. Drug dosage was reduced to between two thirds and one half of the initial dose if severe neutropenia and/or thrombocytopenia occurred between courses. When neutropenia and/or thrombocytopenia persisted, treatment was delayed until granulocyte and platelet counts had recovered. Treatment was continued indefinitely or until relapse occurred.

Supportive Care

Patients were treated in an open hospital ward. Fever was investigated with appropriate cultures and X-rays, and immediate antibiotic therapy was started. Platelet concentrates (4–8 units) were administered two to three times weekly to patients with severe thrombocytopenia (< 20,000 platelets/mm³); in the presence of active bleeding, daily platelet transfusions were given.

Patient Evaluation

Initial laboratory studies included peripheral blood counts, bone marrow aspiration for morphology, and serum muramidase levels. During remission-induction and consolidation therapy blood counts were performed daily for inpatients and twice weekly for outpatients; bone marrow aspirations were done before each course of CAV. During maintenance therapy blood counts were initially performed at 2-week intervals, but later the counts were done less frequently; bone marrow aspirations were performed before each course of BCNU/cyclophosphamide and more frequently if indicated.

RESULTS

Remission-Induction and Consolidation Treatment

The overall results of remission-induction treatment are included in table 1. Thirteen of 29 patients achieved CR and one achieved a good partial remission. The total remission rate was therefore 48%. The median age of the 13 patients who achieved CR was 37 years (range, 16–66 years). Fifteen patients with a median age of 52 years (range, 19–81 years) failed to respond to treatment; seven of these died within 5 weeks of diagnosis and were classified as "early deaths."

Table 2 shows remission-induction and consolidation data relating to the 13 CR patients; all went successfully through consoli-

[*] Muramidase determinations were kindly done by Dr. Waldemar Pruzanski of the Wellesley Hospital, Toronto, Canada, by the lysoplate method of Osserman and Lawlor (5).

TABLE 1.—Patient characteristics

Patient No.	Age/sex	Diagnosis*	Blast cells and promyelocytes in bone marrow at diagnosis (%)	Serum muramidase level at diagnosis (μg/ml)†	Results of treatment with CAV‡	Survival from diagnosis (wks)
1	16/F	AMML	50	ND	Complete remission	>92
2	17/F	AML	22	6.4	Complete remission	>158
3	19/M	AML	49	9	Failure	35
4	22/F	EL	39	5	Complete remission	61
5	23/M	AML	87	ND	Complete remission	106
6	28/F	AML	65	15.4	Failure	14
7	29/F	APL	79	5.1	Complete remission	34
8	30/M	AML	94	ND	Failure	20
9	30/M	AMML	37	19.8	Partial remission	137
10	31/F	AMML	87	14.3	Complete remission	>114
11	37/M	AML	>95	ND	Complete remission	69
12	38/F	AML	94	ND	Complete remission	>114
13	40/F	AMML	85	ND	Early death	3
14	40/F	AML	90	ND	Early death	3
15	41/F	AML	60	ND	Early death	2
16	47/M	AML	84	11.0	Failure	12
17	47/F	AML	52	15.8	Complete remission	>56
18	52/F	AML	28	ND	Failure	14
19	52/M	AMML	91	ND	Failure	9
20	54/F	APL	90	13.7	Complete remission	>59
21	55/M	AML	27	ND	Complete remission	>168
22	57/M	APL	92	4.9	Early death	2 days
23	58/F	AML	88	10.3	Failure	22
24	59/M	EL	80	8.4	Failure	8
25	60/F	AML	24	13.8	Early death	5
26	65/M	APL	41	15.5	Complete remission	>115
27	65/F	APL	49	ND	Early death	2
28	66/M	AML	48	5.8	Complete remission	>66
29	81/M	AMML	77	36	Early death	1 day

*AML = acute myelocytic leukemia; AMML = acute myelomonocytic leukemia; APL = acute promyelocytic leukemia; EL = erythroleukemia.

† ND = not done. Normal values = 5–15 μg/ml of serum.

‡ CAV = cyclophosphamide, cytosine arabinoside, and vincristine.

TABLE 2.—Remission characteristics

Patient No.	Remission induction		Consolidation		BCNU/ cyclophosphamide maintenance		Duration of 1st remission (wks)	Survival from diagnosis (wks)	Comments
	No. of CAV* courses	Treatment (wks)	No. of CAV courses	Treatment (wks)	No. of courses	Treatment (wks)			
1	4	12	6	16	5	>42	>80	>92	Alive and well
2	2	9	2	7	3	47	54	158	Re-induced and maintained with CAV; alive and well
4	4	12	9	23	3	22	45	61	Died 4 wks after relapse without further therapy
5	5	12	5	17	5	37	54	106	Died 40 wks after relapse without further therapy
7	4	9	4	11	2	14	25	34	Died within 24 hrs of relapse with disseminated intravascular coagulation
10	2	5	6	15	8	>72	>107	>114	Alive and well
11	4	10	6	17	2	16	33	69	Died 26 wks after relapse, after failure of CAV re-induction
12	5	14	8	21	7	>57	>100	>114	Alive and well
17	3	9	2	6	3	>19	43	>56	Alive and well
20	2	5	3	11	3	>21	>53	>59	Alive and well
21	4	11	2	8	5	>127	>157	>168	Alive and well
26	3	8	6	17	9	>68	>107	>115	Alive and well
28	4	9	4	13	3	>22	>57	>66	Alive and well

*CAV = cyclophosphamide, cytosine arabinoside, and vincristine.

dation therapy and were then given BCNU/ cyclophosphamide maintenance therapy.

Table 2 also provides data concerning maintenance therapy. By the end of November 1974, six of the 13 CR patients had relapsed. Of these, one did not return to this hospital and died 4 weeks later without further therapy, one died within 24 hours of the diagnosis of relapse with disseminated intravascular coagulation, and one survived another 40 weeks without treatment. Three patients were re-treated with CAV; one improved temporarily but died 26 weeks after relapse, one achieved a second CR and is being maintained with repeated courses of CAV 95 weeks after relapse, and one is undergoing remission induction with CAV.

Survival and Duration of CR

The median survival from diagnosis for the 29 patients included in the study was 34 weeks, ranging from 1 day to greater than 158 weeks. Those patients who failed to achieve CR had a median survival of 8 weeks (range, 1 day to 35 weeks). The median survival of the 13 patients who achieved CR cannot be determined because only four have died. The median survival of these patients is at least 92 weeks and can be projected to be 144 weeks.

The minimum median duration of remission is 54 weeks and the median duration of remission may be as long as 65 weeks.

Figure 1 shows the typical course of one patient who is still in remission (patient No. 10).

Toxicity of BCNU/Cyclophosphamide Maintenance Treatment

Maintenance therapy with BCNU/cyclophosphamide was associated with two types of toxicity. In addition to overnight nausea and vomiting directly after drug administration, patients usually showed significant but transient falls in the blood levels of their platelets and granulocytes between courses. These effects were accompanied by bone marrow hypoplasia. Usually two nadirs in the peripheral blood counts were detected: the first occurred at 10–15 days and was attributed to the high dose of cyclophosphamide; the second occurred at 30–40 days and was probably caused by BCNU. Two of the patients received platelet transfusions but only for a limited time because counts recovered quickly. Transfusions of packed red cells were required for treatment of anemia in only one patient.

A second unique type of toxicity affected the recovery of platelets and granulocytes. While recovery was initially rapid, the rebound to normal levels tended to take a longer time with each successive course of BCNU/cyclophosphamide in some patients. Figure 2 illustrates such a patient. Successive peripheral doubling times of platelets were measured as 5, 12, 15, and 42 days; similar delays in granulocyte recovery were also observed. After six courses of BCNU/cyclophosphamide, recovery was so delayed that no further treatment was adminis-

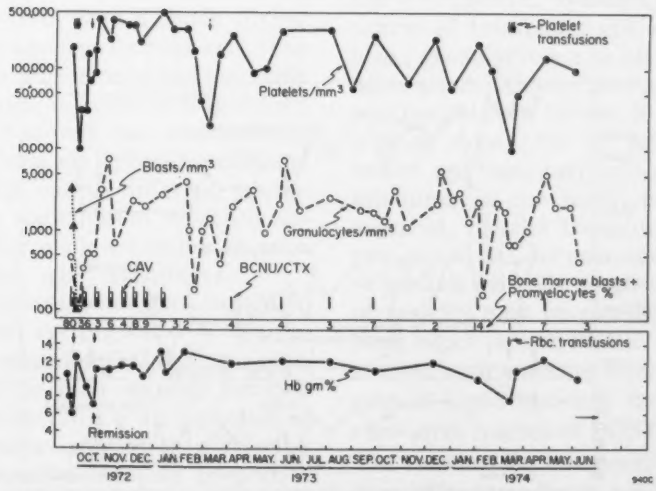

FIGURE 1.—Clinical course of patient No. 10. Therapy, marrow blasts and promyelocytes, hemoglobin, blood blast cells, granulocytes, and platelets are shown. The rise in marrow blast cells and promyelocytes to 14% at the end of January 1974 was thought to represent a shift to the left with marrow regeneration following therapy, since the patient has remained in remission.

tered. The patient is still in remission 84 weeks after his last treatment (patient No. 21).

This delayed recovery of hemopoietic elements may also have affected levels of blast cells in the bone marrow in two of the relapsing leukemic patients. In patient No. 5 (fig 3), the increase of blast infiltration in the marrow was very slow; moreover, a significant degree of myelofibrosis became apparent. The patient did not wish further treatment and died 40

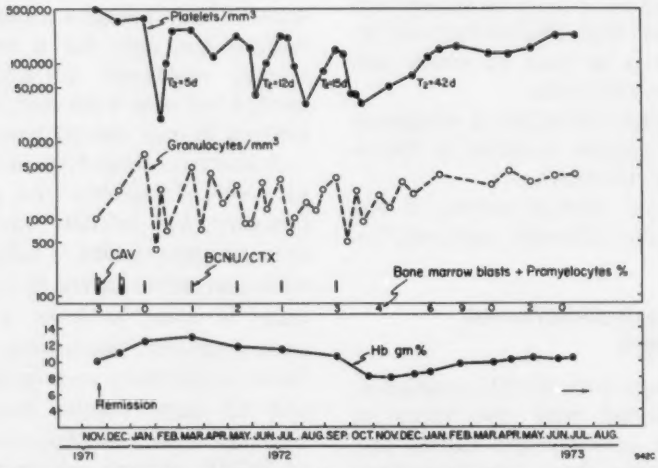

FIGURE 2.—Clinical course of patient No. 21 from the start of CR. T_2 represents the platelet count doubling time in days. Because of the slow recovery of platelets and granulocytes after the 5th course of BCNU/cyclophosphamide (CTX), no further therapy has been given. This patient continues in CR.

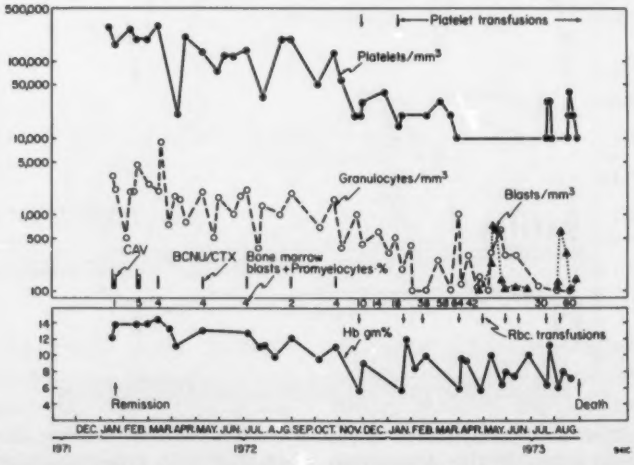

FIGURE 3.—Clinical course of patient No. 5 from the start of CR. Marrow relapse occurred in November 1972, and thereafter there was a very slow increase in the proportion of marrow blast cells and promyelocytes.

weeks after bone marrow relapse. Patient No. 2 was treated with CAV 1 month after a slow increase in marrow blast cells was observed and she achieved remission.

DISCUSSION

Maintenance of remission in acute nonlymphoblastic leukemia is being attempted in a variety of ways. Most often the same treatment that was used during remission induction is pursued during remission, usually at a lower dose and/or at greater time intervals. For example, Whitecar et al (8) use 5-day courses of vincristine, prednisone, cyclophosphamide, and cytosine arabinoside both as induction treatment and as maintenance treatment. On the other hand, Clarkson (9) maintains remission with several chemotherapeutic agents, different from those used in remission induction, administered in a definite sequence over 2-month periods. Finally, others (10,11) are trying to maintain remission by a totally different mode of treatment, introducing immunotherapy at this stage of the disease.

The drug combination described in this paper originated from experimental work done by Bruce et al (13,14). These researchers have previously shown that cyclophosphamide, 5-fluorouracil, and BCNU have a much higher toxicity for lymphoma colony-forming cells than for hemopoietic stem cells in mice and markedly prolong the life of mice injected with lymphoid cells. Moreover, they showed that resistance of lymphoma stem cells to cyclophosphamide and BCNU was probably due to different mechanisms and that the combination markedly increased the cell kill, curing a number of mice bearing transplanted AKR lymphoma.

BCNU and cyclophosphamide are thought to kill both cycling and resting cells. While the latter drug clearly acts as an alkylating agent, the effect of BCNU has been variably ascribed to alkylating properties (15), to an inhibition of DNA repair of lesions induced by alkylating agents (16), to vasculitis-inducing properties (17), or to a modification of the proliferative characteristics of cells (18).

The results of this study show that the combination of BCNU and cyclophosphamide effectively maintains patients with acute nonlymphoblastic leukemia in remission. Our results, expressed in terms of remission and survival duration, compare favorably with those reported in the literature (table 3).

In addition to the therapeutic effectiveness of BCNU/cyclophosphamide in maintaining remission, several other advantages of this drug combination should be emphasized. Foremost is the ease of treatment. The patient is only required to come to the hospital overnight every 8 weeks at the most. During the interim, patients enjoy an entirely satisfactory and active life. This contrasts sharply with most other modes of remission maintenance, which often require repeated sojourns in the hospital, or alternatively, great expenditure of time both by the patient and by the medical and nursing staff. A second benefit accrues from the fact that the patient remains sensitive to the initial remission-induction drug scheme, which can then be used again in case of relapse. This is especially important since only a small number of agents are effective in remission induction.

Myelotoxicity associated with BCNU/cyclophosphamide falls into two categories. There are first transient changes affecting the levels of platelets and granulocytes as a consequence of the administration of two drugs, both toxic to the hemopoietic system. Often, following a course of treatment, one notes two nadirs of blood granulocytes and platelets; one at 10–15 days, attributable to the cyclophosphamide, and another between 30 and 40 days due to BCNU, a drug known for its delayed toxicity. Both nadirs are transitory and seldom need medical attention. In our study, no serious infections occurred, platelet transfusions were given on two occasions, and only once was a transfusion of packed red cells necessary. The second type of toxicity may be specific for drug combinations which include BCNU. Some of our patients showed a progressive slowing of the recovery rate of blood platelets and granulocytes with successive courses of BCNU/cyclophosphamide. The reasons for this peculiar behavior are not clear. The increase in cell-cycle time in L1210 cell cultures treated with BCNU, reported by DeVita et al (18), may be relevant. The unknown mechanism(s) responsible for this increase in cell-cycle time may play a role in

TABLE 3.—Comparison of remission duration and survival of acute nonlymphoblastic leukemia patients treated with various forms of maintenance therapy

Investigators (ref. No.)	No. of CR/total No. of patients treated	Maintenance therapy	Median duration of CR (wks)	Median survival of CR patients from diagnosis (wks)
Whitecar et al (7) (acute nonlymphoblastic leukemia patients only)	17/39	COAP, 5-day course q21d	42	—
Gluckman et al (12)	16/30	Cytosine arabinoside, 5-day course q24d; re-induction with cytosine arabinoside + daunorubicin frequently	52*	—
Powles et al (10)	42/107	19 patients: cytosine arabinoside + 6-thioguanine, 5-day course alternating with cytosine arabinoside + daunorubicin, 5-day course q28d	27	52
		23 patients: same drugs as above + bacillus Calmette-Guérin and leukemic cells	45	86
Southeastern Cancer Study Group (11)	75/206	23 patients: methotrexate twice weekly	26	—
		18 patients: bacillus Calmette-Guérin followed by methotrexate twice weekly	39	—
Present study	13/29	BCNU/cyclophosphamide, q8wks	>54*	>92*

*Minimum median interval calculated from actuarial curves.

the delayed hematologic recovery observed in some of our patients following prolonged treatment with BCNU/cyclophosphamide, the prolonged remissions, and the slow increase in the percentage of leukemic blast cells in the marrow of two relapsing patients. The fact that one of these patients lived for over 40 weeks without treatment after relapse, while the other achieved a second remission following CAV treatment, militates against interpreting this phenomenon in a deleterious sense.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CAV = cyclophosphamide, cytosine arabinoside, and vincristine

COAP = cyclophosphamide, vincristine, cytosine arabinoside, and prednisone

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl) methyl]-methylamino]benzoyl]-
Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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ADDENDUM

When galley proofs were reviewed on March 26, 1975, eight of 13 patients had relapsed. Median duration of complete remission is 88 weeks. Seven of the patients have died. The estimated median duration of survival is 102 weeks.

Fate of Streptozotocin (NSC-85998) in Patients With Advanced Cancer [1,2,3]

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SUMMARY

We have investigated the distribution, biotransformation, and excretion of streptozotocin and its ^{14}C- and ^{3}H-labeled metabolites in 15 patients with advanced cancer. Streptozotocin was detected in the plasma during the first 3 hours after administration while radioactive products were present for longer than 24 hours. No unchanged streptozotocin was detected in the cerebrospinal fluid (CSF); however, ^{14}C-labeled metabolites were detected in the 2-hour CSF sample in a concentration equivalent to the 2-hour plasma level. ^{3}H activity in the CSF was not detected at this time period. Radioactivity measured in biopsied tissues indicated that streptozotocin labeled with ^{14}C and ^{3}H or its metabolites penetrated tumor tissue. ^{14}C tissue levels were found to approximate plasma levels; however, ^{3}H levels were found to be greater than the corresponding plasma levels. Fifteen percent of the total dose of streptozotocin administered was recovered in the urine. ^{3}H-labeled metabolites were recovered in excess of 60% in the urine, and approximately 30% of the ^{14}C-labeled metabolites were recovered in the urine during a similar interval. Less than 1% of the administered ^{14}C and ^{3}H was recovered in the feces. ^{14}C-labeled CO_2 was also recovered, although quantitative recovery was not attained. At least three major metabolites of streptozotocin were detected in the urine by radiochromatography. Two metabolites contained only ^{3}H and one metabolite contained both ^{14}C and ^{3}H in the same ratio as administered.

[Cancer Chemother Rep 59:547–556, 1975]

The antibiotic streptozotocin was first isolated by investigators at Upjohn Research Laboratories from the fermentation broth of *Streptomyces achromogenes* var. 128 (1). Initial in vitro studies indicated that the compound was active against both gram-positive and gram-negative organisms (2). Further investigation indicated that streptozotocin inhibited DNA synthesis in both bacterial and mam-

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[2] Supported by Division of Cancer Treatment (DCT) c ntract NIH-70-2136 and Public Health Service grant CA-06071 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Streptozotocin was supplied by Cancer Therapy Evaluation, DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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malian cells (3). The response against L1210 leukemia prompted the clinical testing of streptozotocin in cancer patients. Since a diabetogenic action was evident during animal toxicity studies, streptozotocin was evaluated for treatment of metastatic insulinoma and was shown to be active against this specific tumor (4). Recently published data on the efficacy of streptozotocin in the treatment of neoplastic disease also indicate that this antibiotic may be effective in malignant carcinoid tumors, Hodgkin's disease, and lymphocytic lymphoma (5–8).

In this report we present the distribution, biotransformation, and excretion of streptozotocin in patients with advanced cancer as part of a phase II study with this drug.

MATERIALS AND METHODS

^{14}C- and ^{3}H-labeled streptozotocin compounds were synthesized by the Monsanto Research Corporation. Chemical and radiochemical purity of the compounds were greater than 95% as demonstrated by radiochromatographic assays. The specific activity of the ^{14}C-labeled material was 6.498 microcuries/mmol, and the specific activity of the ^{3}H-labeled compound was 37.565 microcuries/mmol. Figure 1 illustrates the structure of streptozotocin and the location of the ^{14}C and ^{3}H labels.

Radiochemical Techniques

Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer (models 3375 or 3310). Relative activity (cpm) was corrected to absolute activity (dpm) by use of an external standard. Paper and thin-layer chromatograms were scanned for radioactivity with a Packard radiochromatogram scanner or cut into strips and counted in a liquid scintillation system-Triton X-100 fluor: 1,4-bis-2-(phenyloxazolyl)-benzene (POPOP), 99 mg; 2,5-diphenyloxazole (PPO), 5.0 g; and Triton X-100, 300 ml diluted to 1000 ml with toluene.

Measurement of radioactivity in non-aqueous samples was obtained by combustion in a Packard Tri-Carb Sample Oxidizer (model 305).

Tissue and fecal samples were weighed, wrapped in Whatman No. 1 filter paper, and

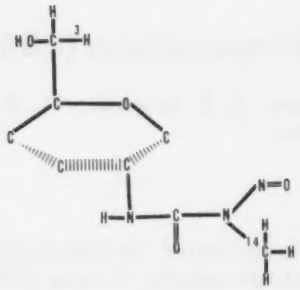

FIGURE 1.—Structure of streptozotocin showing the placement of the ^{3}H and ^{14}C labels.

combusted. Aqueous samples (ie, whole blood, plasma, and tissue homogenate) were pipetted into cotton balls 1–2 cm in diameter prior to combustion. The scintillation mixture used for counting ^{14}C was Permablend III fluor (PPO, 15 g, and *p*-bis-(*O*-methylstyryl)-benzene, 1 g; diluted to 1000 ml with toluene). The scintillation mixture used for counting ^{3}H was Permafluor II (PPO, 5 g, and dimethyl-POPOP, 100 mg; diluted to 1000 ml with toluene).

Chromatography

Descending paper and ascending thin-layer chromatography were performed with Whatman No. 1 paper and Avicel F plates (Analtech) respectively in three solvent systems (all proportions vol/vol): *n*-butanol:acetic acid:water (4:1:1); *n*-butanol:pyridine:water (6:4:3); and isopropanol:water (4:1). After development, radioactivity on the chromatograms was located by either direct scanning or counting 1-cm sections of the chromatogram in Triton X-100 fluor.

High-speed analytic liquid chromatography was used to determine unchanged streptozotocin in plasma and urine. A column (2 mm × 100 cm) of 3,3′-oxypropionylnitile (Durapak) was used with a hexane:isopropanol (3:1) solvent system. Plasma was diluted 1:1 with absolute ethanol and centrifuged, and 5 μl of 6N HCl was added to 2 ml of the supernatant to adjust the pH to 4.0. A lyophilized aliquot of urine was taken up in methanol:acetone (3:1) and centrifuged in order to precipitate urinary salts. Ten μl of supernatant from the plasma or urine was injected into the liquid chromatograph.

The area under the peak absorption band (254 nm) was used to quantitate the level of unchanged drug.

Colorimetric Techniques

An adaptation of the Bratton-Marshall procedure was also used for the colorimetric detection of unchanged streptozotocin (9). The reaction produced a magenta color and the absorption was measured at 550 nm on a Beckman DU spectrophotometer. A minimum of 3 μg/ml of streptozotocin was detectable by this procedure. Deviation from linearity was found to exist above 90 μg/ml. Urinary levels of unchanged drug were measured by the same method. The urine, however, contained substances which interfered with color production and necessitated a dilution of 1:20 with distilled water. Blanks were prepared from plasma and urine samples taken prior to drug administration.

Patient Studies

Patient studies were performed in accordance with the guidelines of phase I and II protocols established by the National Cancer Institute. All patients had advanced malignancies, no renal or hepatic impairment, and no co-existing disorder (such as an infectious process). A minimum duration of 4 weeks elapsed between prior surgery, radiotherapy, or chemotherapy and this study. In all instances, the details of the study and the procedure were explained to the patient and informed consent was obtained.

Streptozotocin was prepared immediately prior to injection by reconstitution of the lyophilized drug with bacteriostatic water. ^{14}C- and/or ^{3}H-labeled streptozotocin was mixed with the unlabeled drug to a total dose of 1.5 g/m^2. The levels of ^{14}C and ^{3}H administered were approximately 100 and 270 microcuries respectively. The drug was administered as a bolus by rapid intravenous (iv) injection through a Millipore Swinnex-25 disposable filter (22μ). Blood samples were collected from the cephalic vein before drug administration, 4 minutes after administration and then every 4 minutes for the next 30 minutes, at 45 minutes, and at 1, 2, 3, 6, 12, 24, and 48 hours. Collections were made in heparinized vacuum tubes that were immediately placed on ice. All blood samples were centrifuged at 2000 rpm for 10 minutes at 0°C as soon as possible after collection. The plasma was decanted and stored on ice until the radioactivity and unchanged drug concentrations were determined.

Levels of radioactivity were measured in the cerebrospinal fluid (CSF) of three patients after administration of ^{14}C- and ^{3}H-labeled streptozotocin. Aliquots of CSF were counted in duplicate in Triton X-100 fluor. Urine was collected from most patients by insertion of a Foley catheter prior to the start of the study. The urine was kept on ice during the collection and the total volume at each sampling interval was recorded. Collections of urine were made at hourly intervals, when possible, for the first 6 hours, and at 12, 24, and 48 hours. Urine samples not used in the assay procedures were immediately lyophilized in 100-ml serum bottles in a Virtis Lyophilizer. The lyophilized urine samples were stored at −10°C until reconstituted for chromatography of urinary metabolic products. Urine samples for immediate use were kept on ice until the completion of the assay procedures. Total fecal material excreted over a 5-day period was obtained from three patients. Feces were stored on ice until homogenization and combustion in a tissue oxidizer for radioactivity determination.

Expired air was collected from four patients who had received ^{14}C-labeled streptozotocin. The patient exhaled through glass tubing into a waste flask and into another flask containing 20 ml of redistilled ethanolamine for 5-minute periods. An aliquot of the ethanolamine (4.5 ml) was mixed with 10 ml of ethanol and 4.5 ml of Packard Permablend III fluor for counting.

Two patients were given both ^{14}C- and ^{3}H-labeled streptozotocin and serial biopsies were obtained over a 5-day period. Biopsy samples were immediately frozen in a dry-ice/acetone bath and were stored at −70°C until examined by tissue combustion.

RESULTS

Eight women and seven men ranging in age from 20 to 73 years were selected for the study.

TABLE 1.—Patients given streptozotocin

Patient No.	Age (yrs)	Sex	Total drug (mg)	Type of streptozotocin given			1° Tumor	Previous therapy
				^{14}C	^{3}H	Unlabeled		
1	60	M	1850			×	Adenocarcinoma of the colon with metastasis to lung and liver	5-FU, NSC-45388
2	28	F	1750			×	Embryonal cell carcinoma with ovarian metastasis to lung and liver	Actinomycin D, methotrexate
3	63	F	1500			×	Carcinoma of pancreas with metastasis to lung and omentum	Intraperitoneal radioactive gold
4	64	F	1075		×	×	Tonsellar lymphoma with liver metastasis	Radiation therapy, methotrexate, procarbazine
5	73	F	2035		×	×	Squamous cell carcinoma of cervix, stage IV at operation	5-FU, cytosine arabinoside
6	66	F	2460		×	×	Leiomyosarcoma of stomach with metastasis to abdominal wall and liver	Cycloleucine, dibromodulcitol, methotrexate
7	65	F	2000	×		×	Epidermal carcinoma of pharynx with metastasis to neck	Radiation therapy, methotrexate, bleomycin
8	57	M	2000	×	×	×	Epidermal carcinoma of gingiva with metastasis to lung, neck, and peritoneum	Radiation therapy, dibromodulcitol, prednisone, procarbazine, methotrexate
9	67	M	2000	×	×	×	Anaplastic carcinoma of lung	Hexamethylmelamine
10	70	M	1800	×	×	×	Malignant melanoma of left leg	Dibromodulcitol, surgery
11	47	F	1870	×	×	×	Ovarian carcinoma with metastasis to liver, heart, adrenal, uterus, bladder; infiltrating ductal carcinoma	Radiation therapy, progesterone derivative
12	72	F	2550	×	×	×	Adenocarcinoma of ovary with metastasis to bone, breast, and pelvis	Hydroxyurea, dibromodulcitol, prednisone, vincristine, methotrexate
13	37	M	2000			×	Malignant melanoma of left neck with metastasis to brain	Cycloleucine, adriamycin, cytosine arabinoside

TABLE 1.—Patients given streptozotocin—Continued

Patient No.	Age (yrs)	Sex	Total drug (mg)	Type of streptozotocin given			1° Tumor	Previous therapy
				^{14}C	^{3}H	Unlabeled		
14	61	M	1500			×	Poorly differentiated epidermoid carcinoma of larynx	Radiation therapy, methotrexate, 6-thioguanine
15	20	M	1500			×	Synovial sarcoma with pulmonary metastasis	Adriamycin, dibromodulcitol, 5-azacytidine

Eight of the 15 patients received ^{3}H-labeled streptozotocin and six patients (including five of the eight patients receiving ^{3}H-streptozotocin) were also given ^{14}C-labeled streptozotocin concomitantly. The remaining six patients received only unlabeled streptozotocin. A summary of the demographic information on the individual patients is presented in table 1.

Plasma Clearance

The mean plasma clearance of radioactivity following administration of ^{14}C- and ^{3}H-labeled streptozotocin indicated that the fall in the level of plasma radioactivity was triphasic and first order (fig 2). The levels of ^{14}C and ^{3}H products were similar during the first 12 hours. The half-life of the initial or α-phase was 6 minutes for both ^{14}C and ^{3}H. The half-life of the second linear segment (β-phase) was 3.5 hours for ^{3}H and 3.2 hours for ^{14}C. The half-life for the terminal linear segment (γ-phase) was greater than 40 hours for both nuclides.

There was no significant difference between colorimetric and high-speed liquid chromatographic determinations of the mean plasma clearance of unchanged drug (fig 3). The half-life of the α-phase was approximately 5 minutes while that of the terminal linear segment (β-phase) was 35 minutes. No unchanged streptozotocin was detected in the plasma by either method after 3 hours following drug administration. However, plasma levels of both nuclides were readily detected at 24 hours.

The transport of labeled drug and metabolites into the CSF was determined in three patients who were given both ^{14}C- and ^{3}H-labeled streptozotocin (table 2). At 1 hour, ^{14}C levels in the CSF were approximately one third of the plasma levels, but at 2 hours, the levels in the CSF were essentially equal to the plasma levels. At 24 hours, ^{14}C activity was greatly reduced in the CSF compared to the plasma level. ^{3}H activity was not detected during the early time intervals; however, at 24 hours the levels were approximately one half of the plasma ^{3}H activity.

Elimination of Streptozotocin and Metabolites

The cumulative percent excretion of ^{14}C and ^{3}H in the urine of nine patients was 20%–30% of the total dose during the first 4 hours after drug administration (fig 4). However, the urinary level of unchanged drug during this period was less than 10% of the total dose. The mean percentage recovery of ^{14}C and ^{3}H during the 24-hour interval accounted for 27% and 57% of the total dose respectively. The recovery of unchanged drug during a similar interval accounted for approximately 11% of the total dose administered.

TABLE 2.—Comparison of ^{14}C- and ^{3}H-activity in CSF and plasma following an iv injection of ^{14}C- and ^{3}H-labeled streptozotocin

Patient No.	Time after administration (hrs)	^{14}C μg equivalents		^{3}H μg equivalents	
		CSF	Plasma	CSF	Plasma
9	1	12.95	35.26	0.0	39.92
8	2	21.07	23.17	0.0	27.91
10	24	0.80	13.67	3.55	6.82

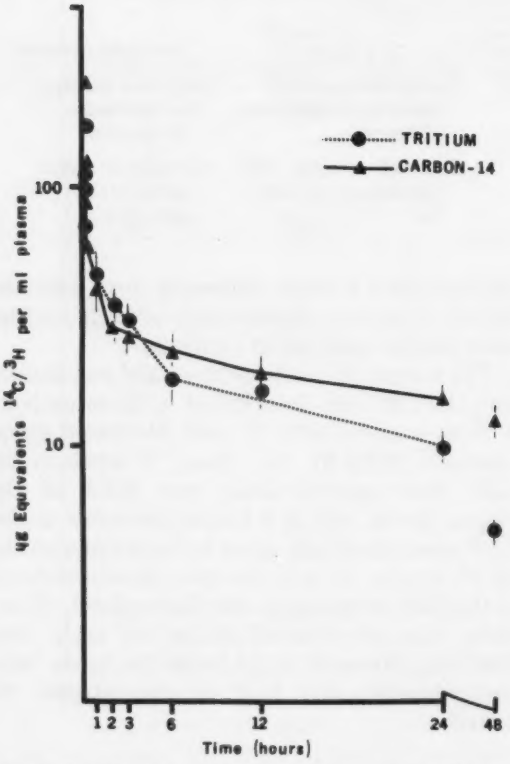

FIGURE 2.—Mean (±SD) plasma clearance of ^{3}H- and ^{14}C-labeled streptozotocin in nine patients. The ordinate is expressed as μg equivalents per ml of plasma, ie, the level of radioactivity resulting from drug and metabolites which is equivalent to 1 μg of unchanged drug.

A comparison of the three different methods used for detection of radiolabeled and unlabeled streptozotocin in urine is presented in table 3. The concentration of unchanged drug detected by the colorimetric method was three times as great as that detected by high-speed liquid chromatography during early collection intervals. During the 4–24-hour collection period unchanged drug could not be detected by the colorimetric method, although it was detected by high-speed liquid chromatography.

Evaluation of other routes of excretion indicated that an insignificant amount of drug or metabolites was excreted in the feces. The total recovery of ^{3}H or ^{14}C in feces was less than 1% of the total administered dose.

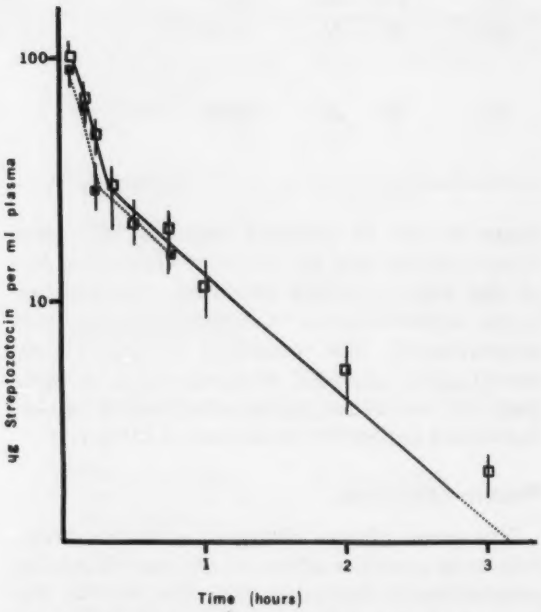

FIGURE 3.—Mean (± SD) plasma clearance of streptozotocin in ten patients.

Although the expired air from four patients given ^{14}C-labeled streptozotocin was not readily quantitated, it was evident that substantial radioactivity was eliminated via this pathway. Approximately 5% of the administered dose was exhaled by one patient during the 24 hours following drug administration. This was calculated from the radioactivity detected in seven half-hour samplings taken over a 24-hour period.

Tissue Levels

Biopsy specimens taken at 1 hour and 4 days (patient No. 11) and at 3 hours, 4 days, and 5 days (patient No. 10) were obtained from two patients who had received both labeled drugs. As a means of comparison, the ratio of the dpm/g of ^{3}H and ^{14}C obtained from each biopsy specimen is compared to the ratio of ^{3}H to ^{14}C administered (table 4). At all periods tested, the levels of ^{3}H remaining in tumor tissue were at least two or three times greater than those of

TABLE 3.—Urinary excretion of streptozotocin and metabolites in patient No. 12 following the administration of ^{3}H- and ^{14}C-streptozotocin*

Time after administration (hrs)	Percent excretion* of streptozotocin labeled with—		Percent excretion* of unchanged streptozotocin detected by—	
	^{3}H	^{14}C	Colorimetric method	High-speed liquid chromatography
0–2	18	15	15	5
2–4	14	5	2	3
4–24	30	8	0	6
Total	62	28	17	14

*Values expressed as percent of total dose administered.

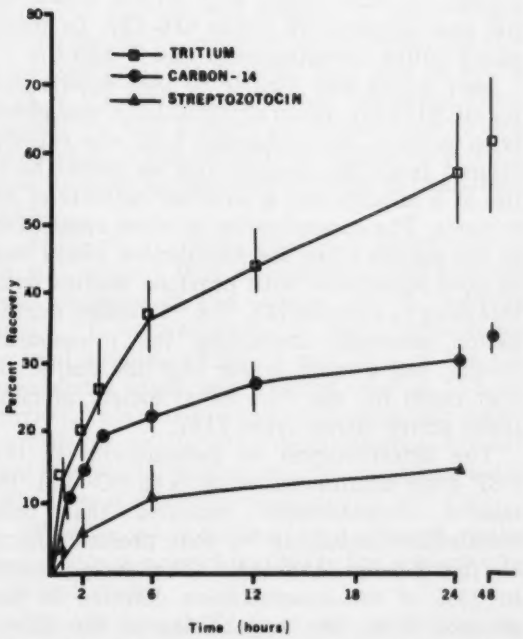

FIGURE 4.—Cumulative percent recovery (± SEM) of unchanged streptozotocin, ^{14}C, and ^{3}H in the urine of nine patients following the simultaneous administration of ^{14}C- and ^{3}H-labeled streptozotocin.

^{14}C. Since the glucose portion of the molecule contained the ^{3}H label, it was this portion which was apparently retained in the tissues. A comparison of the radioactivity in biopsy specimens with corresponding plasma levels indicated that ^{3}H and ^{14}C levels in tumor tissue were either equal to or greater than the levels of radioactivity found in the plasma during the early time intervals. However, by 4 and 5 days the level of ^{3}H far exceeded the level in plasma, whereas ^{14}C remained approximately equal to the plasma level.

Urinary Metabolites of Streptozotocin

Three ultraviolet-absorbing peaks were detected by high-speed liquid chromatography in the urine of patients treated with streptozotocin. Urinary excretion products which absorbed at 254 nm eluted with the solvent front. Unchanged streptozotocin was retained for 8.5 minutes while a metabolite of streptozotocin had a retention time of 15 minutes.

The metabolites of streptozotocin in human urine following iv administration were also examined by paper and thin-layer chromatography (fig 5). Unchanged drug was located at R_f 0.72 and at least four metabolites were recovered in the urine of this patient at the following R_f values: 0.80, 0.58, 0.54, and 0.21. Unchanged drug and the metabolite at R_f 0.54 contained ^{3}H and ^{14}C in the same ratio as administered. The compounds at R_f 0.58 and 0.21 were labeled with only ^{3}H and the compound at R_f 0.80 was labeled with only ^{14}C. When the paper chromatograms from human urine were analyzed by various colorimetric reagents for sugars, positive colorimetric spots indicating the presence of a sugar moiety were obtained from silver nitrate reagent at R_f 0.72 (unchanged drug), R_f 0.54, and R_f 0.21. No evidence for the presence of phosphorylated sugars could be obtained by the use of a molybdic acid rea-

TABLE 4.—Biopsy levels of ^{3}H and ^{14}C after administration of ^{3}H- and ^{14}C-streptozotocin

Patient No.	Time after administration	dpm/g of—		Ratio of $^{3}H/^{14}C$ administered
		^{3}H	^{14}C	
11*	0	—	—	3.62
	1 hr	17,700	2050	8.63
	4 days	15,500	430	36.04
10†	0	—	—	3.41
	3 hrs	16,700	1400	11.93
	4 days	11,900	600	19.93
	5 days	5,890	260	22.65

*Ovarian carcinoma with metastasis to the breast.
†Malignant melanoma of the leg.

gent. Colorimetric analysis for the presence of a nitroso group with the Forist reaction or with aniline-diphenylamine reagent produced positive spots at R_f 0.72 (unchanged drug) and R_f 0.54.

DISCUSSION

The nitrosoureas are known to be an unstable class of chemical compounds. 1-Methyl-1-nitrosourea, BCNU, and streptozotocin decompose rapidly in vitro unless kept within a narrow pH and temperature range (10–12). In phosphate buffer, streptozotocin had a half-life of 1 hour which was similar to that determined for BCNU (13). When streptozotocin was given iv to patients, the unchanged drug was rapidly cleared from the plasma with an initial half-life of 5 minutes and a terminal half-life of 35 minutes. The concentration of drug remaining in the plasma after the distribution phase was in good agreement with previous studies with this drug in patients (5). The ^{14}C-labeled degradation products, containing the nitrosourea moiety, had a much longer half-life similar to that noted for the chloroethyl moiety of clinically active nitrosoureas (14).

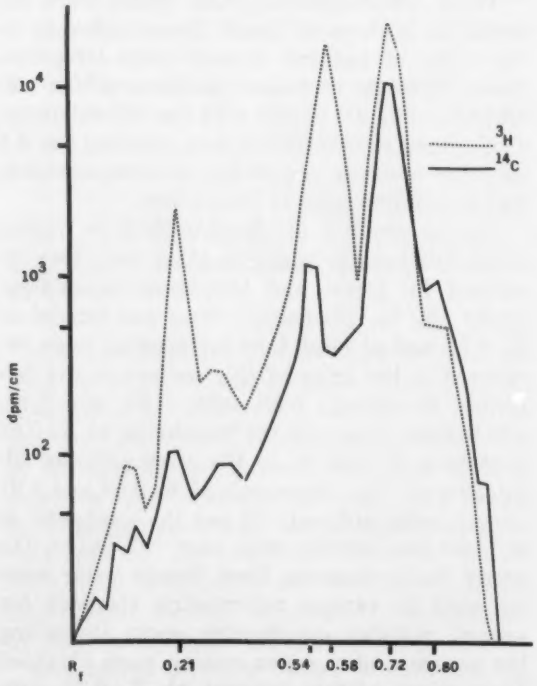

FIGURE 5.—Radiochromatogram of the 0–2-hour urine from a single patient (No. 10) given ^{14}C- and ^{3}H-streptozotocin simultaneously. The solvent system was *n*-butanol:pyridine:water (6:4:3).

The determination of radioactivity in the CSF after administration of both ^{14}C- and ^{3}H-labeled streptozotocin indicated that only metabolites containing ^{14}C were present during the first 2 hours. At this time they were present in 95% of the concentration detected in the plasma. Thus, the metabolites of the nitrosourea portion of streptozotocin enter the CNS much more readily than those of BCNU or CCNU (14). However, whether these metabolites are as active against CNS tumors as those of CCNU remains to be determined. Since both ^{3}H and ^{14}C were present in the 24-hour specimen of CSF, the possibility exists that unchanged drug may also have crossed the blood-brain barrier after the 2-hour period. However, the presence of intact streptozotocin in the CNS has not been demonstrated in several animal experiments (15,16).

The ^{14}C levels in the biopsy specimens approximated the levels in the plasma. However, the ^{3}H levels in these tissues were much higher than their corresponding plasma levels. Although the possibility exists that unchanged drug was present, it was obvious that most of the activity was due to the presence of metabolites as indicated by the increasing ratio of ^{3}H to ^{14}C in biopsy tissues with time. Since the ^{3}H label was located on the glucose moiety, the ^{3}H metabolites would probably be glucose analogs, and as such, they may have been incorporated into tumor cells. It has been demonstrated that D-glucosamine as well as several other hexosamines cause cytoplasmic and nuclear changes in several transplantable tumor lines (17,18). Since ^{3}H activity was present in tumor tissue long after the ^{14}C activity had declined, the glucose portion of the molecule may play an active role in the cytotoxic action of the drug. Thus, it may be this portion of the molecule which confers on streptozotocin its distinctive activity and toxicity as compared to other nitrosoureas (4,6).

Although we were not able to quantitate it, it was evident that considerable radioactivity was excreted in the expired air by patients given ^{14}C-streptozotocin. In more controllable animal studies, we found that 30% of the administered label was expired over a 24-hour period. This was equivalent to what was determined for ^{14}C-1-methyl-1-nitrosourea (14,20) but was much less than was found after administration of ^{14}C-ethylene-labeled CCNU (19).

Whether or not all of the expired radioactivity was present in CO_2 is the subject of a current study.

The two methods used for detecting unchanged streptozotocin were not in close agreement. The colorimetric method indicated that 15% of the drug was present in the urine by 2 hours, whereas only 5% of the administered dose was detected during this period by high-speed liquid chromatography. This discrepancy may be due to the presence of a metabolite containing the nitroso group in the urine. The metabolite located at R_f 0.54 on the paper chromatograms absorbed in ultraviolet light and also gave colorimetric evidence for the presence of a nitroso group. No unchanged drug was detected colorimetrically during the 4–24-hour interval. However, 6% of the administered drug was detected during this period by liquid chromatography. A possible explanation for this difference may be the 20-fold dilution of the urine used for colorimetric analysis and the greater sensitivity of the detection method used for the chromatography.

Because streptozotocin has a methyl group in place of the chloroethyl group of BCNU, the nitrosourea moiety cannot be metabolized to an oxazolidine intermediate as postulated for that drug (13). Nevertheless, it was evident from our studies that although the methyl group in streptozotocin is readily degradated metabolically, it can also be incorporated into a highly lipid-soluble metabolite similar to that formed from BCNU. The cleavage of all or part of the nitrosourea moiety results in the formation of the two ^{3}H-labeled deoxyglucose metabolites detected in the urine.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2-(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cycloleucine: NSC-1026; CAS reg. No. 52-52-8; 1-aminocyclopentanecarboxylic acid

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Streptozotocin: NSC-85998

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Multiple Myeloma Resistant to Melphalan (NSC-8806) Treated With Cyclophosphamide (NSC-26271), Prednisone (NSC-10023), and Chloroquine (NSC-187208) [1,2,3]

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SUMMARY

A prospective randomized study of the effects of two different therapeutic regimens was conducted in 41 patients with multiple myeloma resistant to therapy with melphalan. The results of treatment with cyclophosphamide plus prednisone were compared with those of cyclophosphamide, prednisone, and chloroquine. Chloroquine, which inhibits the repair process of DNA in animals, has reportedly produced responses to alkylating agents to which the animal had been resistant. No significant differences in response to the doses used with either regimen could be found. Toxicity consisted mainly of leukopenia and thrombocytopenia.

[Cancer Chemother Rep 59:557–562, 1975]

Melphalan is well established in the treatment of multiple myeloma (1–4). Resistance to melphalan often develops in patients with an initially satisfactory response. In addition, some patients do not respond to melphalan even initially. Cyclophosphamide has also been used with beneficial results by many chemotherapists in the treatment of myeloma (5–7), and patients whose condition is resistant to melphalan have shown improvement when treated with cyclophosphamide (8). Evidently, resistance to one alkylating agent does not

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[2] This study was done by the Acute Leukemia Group B (ALGB) (James F. Holland, chairman) and was supported in part by Public Health Service grants CA-04646, CA-07968, CA-12886, CA-02599, and CA-05834 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Melphalan: CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Prednisone: CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

Chloroquine: CAS reg. No. 54-05-7; 1,4-pentanediamine, N^4-(7-chloro-4-quinolinyl)-N^1,N^1-diethyl-.

Drugs used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[10] The cases were contributed as follows: *Mayo Clinic* (11 patients); *State University of New York, Downstate Affiliated Hospitals* (8 patients); *Roswell Park Memorial Institute* (6 patients); *New York Hospital* (5 patients); *Bowman Gray School of Medicine* (2 patients); *Medical College of Virginia* (2 patients); *Long Island Jewish Hospital* (1 patient); *Mt. Sinai Hospital* (1 patient); *Basel University Clinics, Switzerland* (1 patient); and *University of Pretoria, Republic of South Africa* (1 patient).

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necessarily mean resistance to a second alkylating agent.

Recent work indicates that chloroquine or caffeine inhibits the repair of DNA in cyclophosphamide-resistant plasmacytomas in hamsters, so that these tumors become responsive to cyclophosphamide, mechlorethamine,[12] or irradiation (9). This work suggests that one mechanism of resistance to alkylating agents may be an enhanced ability to repair the damage caused by alkylating agents on DNA.

The objectives of this study were to determine whether patients with myeloma who manifested resistance to melphalan would respond to cyclophosphamide and whether chloroquine augments this response.

MATERIALS AND METHODS

Criteria

All patients fulfilled the criteria for the diagnosis of multiple myeloma (10). They were all resistant to melphalan as demonstrated by the lack of response after an adequate therapeutic trial of 3 months or progression of myeloma after an initial response to adequate doses of melphalan. Progression was characterized by an increase in the M protein in the serum, an increased M spike (globulin) in the urine, a decrease in hemoglobin concentration, an increase in the proportion of plasma cells in the bone marrow, a progression of bone lesions, an increase in pain, or a decrease in performance status. Patients entered in the study had to have an estimated survival of at least 2 months, a serum calcium value of less than 12 mg/dl, and no significant infection. Previous radiation therapy did not disqualify the patient, but irradiation must have been completed at least 6 weeks before the patient entered the study. If corticosteroids were given they must have been administered as a constant low maintenance dose only. Patients were not randomized until recovery from drug-induced cytopenia had occurred. Finally, patients with other disorders characterized by plasmacytosis in the bone marrow such as metastatic carcinoma, connective tissue diseases, chronic infections, and cirrhosis were excluded.

[12] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

Grading

The status of each patient was rated arbitrarily from 0 to 4: 0 = the patient's activities were normal; 1 = the patient had mild symptoms but was ambulatory; 2 = the patient had moderately severe symptoms but was in bed less than 50% of the waking time; 3 = the patient had severe symptoms and was in bed more than 50% of the waking time; and 4 = the patient was completely bedridden.

Initial Investigations

The following were included in the initial evaluation: history and physical examination; determinations of hemoglobin concentration or hematocrit value; determinations of leukocyte (total and differential), platelet, and reticulocyte counts; determinations of values for blood urea nitrogen or creatinine, blood uric acid, and serum calcium; serum protein electrophoresis; urinalysis, examination of urine for Bence Jones proteinuria, total protein concentration, and analysis of a 24-hour urine specimen by electrophoresis; studies of bone marrow aspirates; a roentgenographic skeletal survey; and funduscopy.

Investigations During Therapy

Every 6 weeks, the following tests were advised: determinations of hemoglobin or hematocrit values and leukocyte and platelet counts; serum and urine electrophoresis; determinations of blood urea nitrogen, creatinine, uric acid, calcium, phosphorus, and alkaline phosphatase values; and bone marrow aspirations. All initial tests were repeated when the study of each patient was terminated. Funduscopy was performed at 3-month intervals.

Evaluation of Response to Therapy

The following definitions of parameters were used for the purposes of evaluation: (*a*) *hemoglobin*, an increase of 2 g/dl or more that persisted for at least 2 weeks in the absence of blood transfusions in patients whose initial hemoglobin concentration had been 11

g/dl or less; (*b*) *hematocrit*, an increase of 5.5% under the same circumstances, the initial value being 35% or less; (*c*) *serum globulin concentration*, a decrease of 2.5 g/dl or more from an initial value of more than 4.5 g/dl, a return to normal values, or a disappearance of the globulin spike; (*d*) *urinary globulin excretion*, a decrease of at least 50% from an initial value of 1.0 g/24 hrs or more; (*e*) *proportion of plasma or myeloma cells*, a decrease of 200 cells/1000 total marrow cells; (*f*) recalcification of bone lesions in the absence of new lesions developing during the study; (*g*) improvement of performance status by two grades; and (*h*) decrease in pain by two grades, pain being graded on the basis of severity from 0 through 4.

Treatment Regimens

Patients who fulfilled the criteria for admission to the study were randomly allocated to one of two treatment regimens. Regimen 1 consisted of the administration of (*a*) 600 mg/m^2 of cyclophosphamide on Day 1 intravenously, and (*b*) 0.8 mg/kg/day of prednisone orally for 14 days, 0.4 mg/kg/day orally for the next 14 days, 0.2 mg/kg/day orally for the next 14 days, and then discontinued abruptly on Day 43. Regimen 2 consisted of the same schedule of cyclophosphamide and prednisone but, in addition, 250 mg of chloroquine was given twice daily beginning 7 days before the injection of cyclophosphamide and continued until 3 days after the start of cyclophosphamide and prednisone therapy. In order to maximize or minimize the effects of caffeine on the repair enzymes, all patients who received chloroquine in regimen 2 were told to drink at least 6 cups of tea, coffee, or cola drinks daily. Those receiving regimen 1 were told to avoid tea, coffee, cola drinks, and other substances containing caffeine.

With both regimens, leukocyte and platelet counts were determined 6 weeks after the initial injection of cyclophosphamide and the patients received another course of therapy. The dose of cyclophosphamide was altered depending on the leukocyte and platelet values as follows: if the leukocytes numbered more than 5000 cells/mm^3, 100% of the dose was given; if 4000–4999, 75% was given; if 3000–3999, 50% was given; if 2000–2999, 25% was given; and if less than 2000, the dose was delayed. If the concentration of platelets was greater than 100,000 cells/mm^3, 100% of the cyclophosphamide dose was given; if 75,000–100,000, 50% was given; and if less than 75,000, the dose was delayed until the platelets had increased. Prednisone was given in a dosage of 0.5 mg/kg for 7 days with each subsequent course.

RESULTS

Of 41 patients entered in the study, two were ineligible because they had not previously received melphalan. One patient was removed from the study on Day 4 because of a preexisting macular degeneration. Of the remaining 38 patients, 20 received regimen 1 and 18 received regimen 2.

The total number of abnormalities in each patient at the start of therapy was virtually the same for both regimens (ie, 5.3 for regimen 1 and 5.6 for regimen 2), which indicated that no significant differences existed before therapy was started.

Responses to the two regimens were essentially similar and were not impressive. Among patients treated with regimen 1, 14 of 105 abnormal parameters initially present responded (13.3% or 0.7 parameter/patient); among patients treated with regimen 2, 15 of 100 abnormal parameters responded (15.0% or 0.8 parameter/patient) (table 1). If the patients who received only one course of therapy are not considered, the number of parameters responding was 1.1/patient (or 20%) for both regimens.

Categorization of responses as good (ie, more than half the initially abnormal parameters responded) or limited (less than half the initially abnormal parameters responded) showed that less than 50% of the patients in either group had any response (table 2).

Examination of individual responses showed a meaningful response in only four of 34 patients with anemia and in only three of 28 with an abnormal concentration of serum proteins (table 1). In nine of 34 patients there was a substantial decrease in the proportion of plasma cells in the bone marrow. In six of 20

TABLE 1.—Response frequencies of laboratory or clinical parameters

Parameter	Regimen 1		Regimen 2	
	No. of initially abnormal parameters	No. responding	No. of initially abnormal parameters	No. responding
Hemoglobin (<11 g/dl)	17	2	17	2
Serum globulin	14	0	14	3
Urine protein (>1 g/24 hrs)	4	1	5	2
Marrow plasma cells (>20%)	19	4	15	5
BUN (>30 mg/dl)	6	0	8	0
Serum calcium (>11 mg/dl)	3	1	3	0
Bone lesions	20	0	12	0
Soft tissue lesions	1	0	2	0
Pain (>2 grades)	9	4	11	2
Performance status (>2 grades)	12	2	13	1
Total	105	14	100	15

TABLE 2.—Frequency of responses in patients treated with regimen 1 or 2

Regimen	No. of patients treated	No. of responses		
		Good	Limited	None
1	20	1	7	12
2	18	1	7	10

patients pain was noticeably relieved, and in only three of 25 patients was there an appreciable improvement in performance status. No bone lesions healed.

The number of courses of therapy varied, but seven patients treated with regimen 1 and four treated with regimen 2 received one course of therapy only. None of these patients showed any response. Four died within 10 days of starting therapy; massive pulmonary emboli accounted for two deaths, rupture of an abdominal aortic aneurysm occurred in one, and a myocardial infarction occurred in another. Only four patients treated with regimen 1 and five patients treated with regimen 2 had more than five courses of therapy. All responses except one were seen in patients who had received three or more courses of therapy. No responses were noted among patients receiving 1 g or less of cyclophosphamide and only one response occurred when the total dose was less than 2 g. Five patients received more than 10 g of cyclophosphamide (table 3).

Toxicity consisted mainly of leukopenia and thrombocytopenia, and there were no significant differences between the two regimens. Leukopenia (a leukocyte count $\leq$ 2000 cells/mm^3) was present in 11 patients and thrombocytopenia (a platelet count $\leq$ 100,000 cells/mm^3) occurred in 23 patients (both groups) (table 4). Infections occurred in five patients treated with regimen 1 and in none of them was the leukocyte count less than 2000 cells/mm^3. Among those treated with regimen 2 there were eight infections; four occurred in patients in whom the leukocyte count was 2000 cells/mm^3 or less at the onset of the infection. No instances of retinopathy were recorded.

All patients but one have been dropped from the study either because of a lack of response to therapy or because of a response followed by progression of disease, as manifested by increased lytic bone lesions, increased fractures,

TABLE 3.—Total dose of cyclophosphamide

Cyclophosphamide dose (g)	Regimen 1		Regimen 2	
	No. of patients	No. of responses	No. of patients	No. of responses
≤1.0	7	0	5	0
1.1–2.0	1	1	1	0
2.1–4.0	4	3	6	6
4.1–6.0	6	7	2	4
6.1–10.0	0	0	1	2
>10.0	2	3	3	3
Total	20	14	18	15

TABLE 4.—Nadirs of leukocyte and platelet counts

Leukocyte count			Platelet count		
	No. of patients on regimen—			No. of patients on regimen—	
Cells × 10^3/mm³	1	2	Cells × 10^3/mm³	1	2
≤1.0	1	0	≤50	7	4
1.1–2.0	4	6	51–100	5	7
2.1–3.0	5	4	101–200	7	6
3.1–4.0	6	5	201–300	1	1
4.1–5.0	3	1	>300	0	0
>5	1	2		—	—
Total	20	18		20	18

increased amounts of protein in the serum or urine, a decreased hemoglobin concentration, an increased proportion of plasma cells in the bone marrow, increased pain, or a poorer performance status. Twenty-two of the patients have died.

DISCUSSION

The treatment of patients with multiple myeloma is difficult and a challenge. Although melphalan is effective, at least one third of the patients do not respond; moreover, in patients responding to melphalan, the condition almost always becomes resistant and a relapse occurs.

Cyclophosphamide is useful and, in some hands, is as effective as melphalan (6,7). Some patients resistant to melphalan will respond to cyclophosphamide (8).

The response of our patients to cyclophosphamide was not nearly as impressive as that noted by Bergsagel and associates (8); in fact, it was quite disappointing. The smaller doses of cyclophosphamide in our patients may account in part for the discrepancy. Also, resistance to melphalan is difficult to define and comparison of the results obtained by different investigators is not always satisfactory.

Despite the encouraging results in animals, the addition of chloroquine and caffeine to the therapeutic regimen of cyclophosphamide and prednisone did not increase the response or produce any other benefits.

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Continuous Intravenous Bleomycin (NSC-125066) Therapy With Vinblastine (NSC-49842) in Stage III Testicular Neoplasia [1,2]

Melvin L. Samuels, Douglas E. Johnson, and Paul Y. Holoye [3,4]

SUMMARY

Twenty-three patients with stage III germinal neoplasia of the testis were treated with a variation of our original vinblastine-bleomycin program. This modification consisted of 0.4 mg/kg of vinblastine given in two fractions on Days 1 and 2 followed by continuous intravenous administration of 30 units of bleomycin in 1000 cc of 5% glucose and distilled water over a 24-hour period for 5 successive days beginning on Day 2. Therapy was repeated every 28–35 days as toxicity permitted. There were 17 responses, nine of which were complete (39%). Eight of the complete responses were in patients with massive disease in whom a low complete response rate was expected. Toxic effects consisted of severe leukopenia in 90%, thrombopenia in 50%, and unexplained transient hyperbilirubinemia in about 30% of the patients. Bleomycin pneumonitis occurred in one patient and resulted in death. Hypertension was a new and unexpected side reaction experienced by four patients. Further trials are indicated since the complete response rate in patients with advanced massive disease appears to be improved.

[Cancer Chemother Rep 59:563–570, 1975]

Prior treatment programs using intermittent twice weekly bleomycin with either large-dose vinblastine or a five-drug combination program (bleomycin, cyclophosphamide, vincristine, methotrexate, and 5-fluorouracil) have yielded a 75% response rate (1,2). Of 70 stage III patients studied, there were 22 complete and 31 partial responses. The survival time of the entire group has been shown to be superior to that of vinblastine used alone and, further, 12 of the complete responders have been alive for more than 150 weeks with no evidence of disease. Thus, the addition of bleomycin to existing programs has significantly improved palliation and survival time.

Drewinko et al (3) have studied the effect of bleomycin on human lymphoma cells in culture. As with most mammalian tumor cells, the survival curve is biphasic; there is an initial steep part of the curve which represents a large cell kill followed by a flat portion which represents the response of the less sensitive lymphoma cells. Using a single large dose of 50 μg/ml of bleomycin, a 10% survival fraction is obtained after 4 hours of treatment. However,

[1] Received Dec 2, 1974; revised Feb 10, 1975; accepted Feb 19, 1975.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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if the exposure time is increased to 35 hours (one cell cycle), the survival fraction falls to 0.06%. These findings suggest that greater cell kill may be obtained in man by continuous administration.

MATERIALS AND METHODS

Twenty-three patients with stage III metastatic testicular neoplasia were entered into this study between July 1, 1973, and July 1, 1974. Table 1 summarizes the pertinent clinical data. Patients receiving prior chemotherapy had usually received actinomycin D or the five-drug combination prior to administration. Patients with extragonadal primary tumors were excluded and will be the subject of a special report. The criteria of Dixon and Moore were used for histologic classification.

Vinblastine was given in a total dose of 0.4 mg/kg split into two fractions on Days 1 and 2. On the second day, usually 6–8 hours after the vinblastine injection, continuous intravenous (iv) bleomycin was started. Thirty units was diluted in 1000 cc of 5% glucose and distilled water and given over a 24-hour period for 5 successive days. The entire procedure was repeated every 28–35 days (as toxicity permitted) for an anticipated five courses.

Upon entry into the study, patients were divided into the following tumor status groups:

a. Minimal pulmonary disease—no more than five metastases in each lung field with maximum diameter less than 2.0 cm;

TABLE 1.—Patient characteristics

Characteristic	
Primary tumor	Testis
Total No. of patients	23
Age (yrs)	
Median	24
Range	18–56
Duration of disease (mos)	
Median	6
Range	1–18
No. of patients with prior therapy	
None	11
Radiation	6
Chemotherapy	2
Radiation + chemotherapy	4

b. advanced pulmonary disease—any mediastinal or hilar mass, pleural effusion, or intrapulmonary mass greater than 2.0 cm;

c. minimal abdominal disease—minimal pulmonary disease plus positive lymphangiogram with *no* ureteral displacement;

d. advanced abdominal disease—palpable mass, liver metastasis, ureteral displacement with or without obstructive uropathy, and inferior vena cava distortion by metastatic nodes.

Patients with only hormonal manifestations, such as gynecomastia with or without elevated human chorionic gonadotropin (HCG) titers, were not encountered in this series.

Objective tumor regression was graded as follows. (*a*) Complete remission: all measurable tumor had disappeared. If there was any suspicious residual nodule or infiltrate, tomograms were obtained. Retroperitoneal disease required a repeat lymphangiogram and if equivocal, retroperitoneal node dissection. Gynecomastia and HCG titers must have returned to normal. (*b*) Partial remission: at least a 50% decrease in the product of the maximum diameter and maximum perpendicular diameter. Survival time was measured from Day 1 of chemotherapy.

RESULTS

Objective tumor regression was noted in 17 of 23 patients and nine of these responses were complete. Table 2 compares response and prior therapy and it can be seen that previous treatment status did not influence response. Thus, there is no cross resistance with actinomycin D, as was true of previous studies. Also, almost all of our patients were in the unfavorable tumor status category of "advanced disease" which usually implies large bulky masses.

Response by tumor type is given in table 3 and by individual patients in table 4. It can be seen that embryonal carcinoma was the predominant cell type encountered and that virtually all patients with this tumor type responded. Embryonal carcinoma is the most frequent Dixon and Moore subtype in stage III studies but class 4 or teratocarcinoma mixed with embryonal carcinoma or choriocarcinoma is usually the next most frequent group. However, there

TABLE 2.—Prior therapy, disease status, and response

	Total No. of patients	No. of patients entering with—		No. of responses			No. of patients dead*
		Minimal disease	Advanced disease	Complete	Partial	No	
Prior therapy	12	1	11	4	4	4	1
No prior therapy	11	1	10	5	4	2	3
Totals	23	2	21	9	8	6	4

*Includes 1 death from acute myocardial infarction, 1 bleomycin interstitial pneumonitis, and 2 tumor deaths.

TABLE 3.—Tumor type and response

Tumor type	No. of evaluable patients	No. of responses
Seminoma	2	2
Embryonal and seminoma	13	12 (7 complete)
Teratoma	1	0
Teratocarcinoma, mixed	2	1 (complete)
Choriocarcinoma with or without embryonal	5	2 (1 complete)

were only two class 4 patients in this series, while choriocarcinoma (Dixon and Moore, class 5), which normally has about a 1% incidence, was the second most common tumor type with a 20% incidence. Class 5 tumors usually carry the most ominous prognosis. Of the five patients with these tumors entered in the study, there is only one complete response which is still being maintained at 51 weeks.

Two of the partial responders have died, one from advanced disease and one from bleomycin pneumonitis, while no deaths have occurred among the complete responders. Only one complete responder has relapsed after three courses (table 4, patient No. 5) because of an 8-month interval after the third course of therapy. He has been re-induced with the same drug program and is currently achieving a near complete remission (fig 1).

TOXIC EFFECTS

As with previous trials, myelosuppression continues to be the major side effect. Severe leukopenia is to be expected in all patients with the nadir at the end of the first week (table 5). No dose adjustments were made for severe leukopenia unless sepsis supervened. Thrombopenia occurred in about 50% of the initial courses and was of brief duration, not requiring platelet transfusions (table 6). Multiple courses did not result in cumulative marrow toxicity. Bleomycin does not appear to increase vinblastine myelosuppression as judged by the magnitude of depression, time to nadir, and recovery time. Septicemia was encountered in three patients and was directly related to severe leukopenia. All had massive abdominal disease and all recovered. The organisms were *Pseudomonas, Escherichia coli,* and *Candida.* Seven patients showed an elevation of serum bilirubin coupled with a fall in hemoglobin concentration after the first course of therapy (table 7). Liver function studies remained essentially within normal limits and there was no associated hepatomegaly. In patient No. 1, table 7, the etiology of the jaundice seemed to be clearly hemolytic but this was certainly not clear for the other patients. Subsequent re-exposure to the same treatment induced recurrent mild transitory icterus. Four patients exhibited a sustained hypertension, usually noted by Day 6 and lasting several days. The usual elevation ranged from 150/90 to 160/100 but in one patient, an elevation to 190/140 occurred on Day 8 and was followed by an acute anterior myocardial infarction. This patient, age 52, died from ventricular fibrillation and a postmortem examination disclosed severe general-

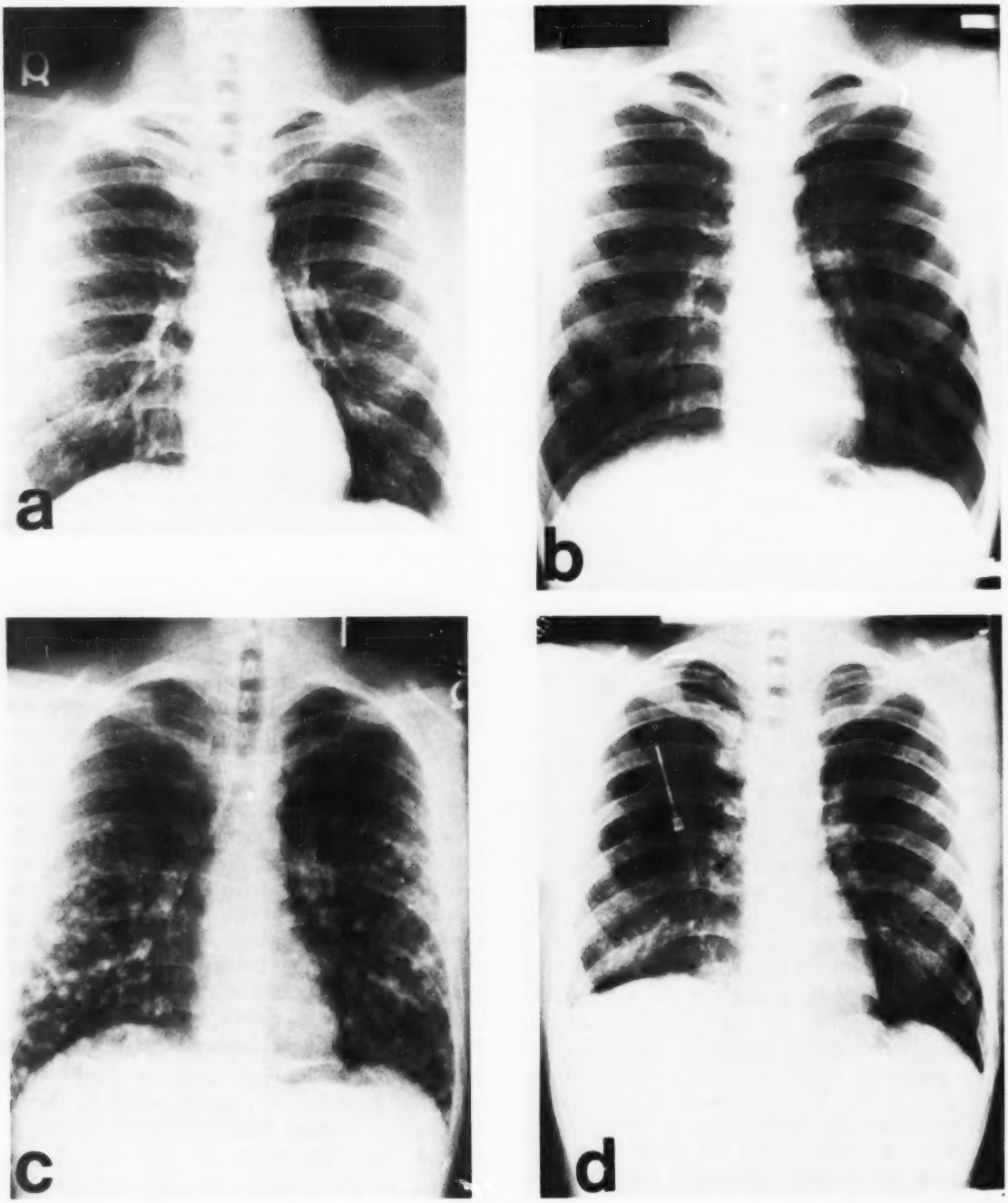

FIGURE 1.—*a*. Mediastinal mass in a 20-year-old man previously treated with radiation and bleomycin + cyclophosphamide + vincristine + methotrexate + 5-fluorouracil; histology is teratocarcinoma + embryonal carcinoma. *b*. After 3 courses of continuous bleomycin therapy. *c*. Recurrent intrapulmonary disease 8 months after third course. *d*. Almost complete remission after reinstitution of bleomycin and vinblastine therapy.

TABLE 4.—Clinical data of individual responders

Patient No.	Age (yrs)	Tumor type	Prior therapy	Disease status upon entry into study	Response	Time to response (wks)	Duration of response (wks)	No. of therapy courses	Survival time (wks)
1	24	Embryonal	Radiation, para-aortic node dissection	Mediastinal and multiple pulmonary nodules, gynecomastia, positive lymphangiogram	Complete	12	20+	5	32+
2	46	Embryonal	Para-aortic node dissection	Small pulmonary nodules (<2.0 cm)	Complete	4	4+	2	10+
3	22	Embryonal	None	Multiple lung nodes, gynecomastia, positive lymphangiogram	Complete	16	24	4	40+
4	29	Choriocarcinoma+ embryonal	None	Positive lymphangiogram, multiple large lung masses, gynecomastia with elevated HCG titer	Complete	12	29+	7	51+
5	20	Teratocarcinoma+ embryonal	Radiation, bleomycin combination*	Mediastinal mass	Complete	8	24 (recurrence)	3	32+
6	20	Embryonal	None	Massive lung metastasis	Complete	8	12+	5	20+
7	20	Embryonal	None	Neck nodes, positive lymphangiogram	Complete†	8	12+	5	20+
8	24	Embryonal	Actinomycin D	Multiple lung masses	Complete	10	14	4	24+
9	24	Embryonal	Radiation, para-aortic node dissection	Small lung nodules (<2.0 cm)	Complete	6	12+	3	18+
10	19	Embryonal	Bleomycin combination*	Bilateral lung masses	Partial	4	12	4	32+
11	37	Embryonal	None	Bilateral lung and abdominal masses, elevated HCG titer, positive α-fetoprotein	Partial	4	8	2	12 (Died)
12	44	Seminoma	None	Huge abdominal mass, positive	Partial	4	20	4	25 (Died)

(*Cont'd*)

TABLE 4.—Clinical data of individual responders—Continued

Patient No.	Age (yrs)	Tumor type	Prior therapy	Disease status upon entry into study	Response	Time to response (wks)	Duration of response (wks)	No. of therapy courses	Survival time (wks)
				lymphangiogram, mediastinal and lung masses					
13	32	Choriocarcinoma	None	Huge abdominal mass, nonfunctioning left kidney, pulmonary masses, supraclavicular nodes, gynecomastia, elevated HCG titer	Partial	6	10+	5	16+
14	46	Embryonal	Radiation	Left supraclavicular mass, recurrent para-aortic disease, lung nodules, positive α-fetoprotein	Partial	4	17	4	52+
15	24	Embryonal	Radiation to abdomen	Huge mass growing in field of radiation (15 × 18 cm)	Partial	2	4	1	8+
16	30	Seminoma	Radiation to abdomen and mediastinum	Massive lung metastases, dyspnea, cyanosis	Partial	2	4	1	8+
17	18	Embryonal	None	Massive lung metastasis, large para-aortic nodes, gynecomastia, negative HCG titer	Partial	4	16	4	24+

*Bleomycin, cyclophosphamide, vincristine, methotrexate, and 5-fluorouracil.
†Confirmed by retroperitoneal dissection.

TABLE 5.—Leukocyte depression

Course No.	No. of trials	No. of patients with wbc count <2000 cells/mm³	Median— Wbc nadir/ range (cells/mm³)	Day of nadir/ range	Day of recovery/ range
1	23	21	1200/600–1900	7/6–11	14/10–21
4	9	9	1000/400–2000	7/6–9	14/12–16

TABLE 6.—Platelet depression

Course No.	No. of trials	No. of patients with platelet count <100,000 cells/mm³	Median— Platelet nadir/ range (platelets/mm³)	Day of nadir/ range	Day of recovery/ range
1	23	12	70,000/29,000–89,000	5/4–11	10/7–14
4	11	9	78,000/33,000–83,000	5/4–7	9/7–14

TABLE 7.—Summary of patients with elevated serum bilirubin

Patient No.	Age (yrs)	Serum bibirubin: Maximum elevation (mg/100 ml)	Serum bibirubin: Treatment day	Serum bibirubin: Duration (days)	Hemoglobin: Maximum depression (mg/100 ml)	Hemoglobin: Day of maximum depression	Remarks*
1	21	7.4 (D-2)†	12	12	−4	9	Haptoglobin, 63 mg/ 100 ml, spherocytosis, negative Coombs' serum
2	19	2.4 (D-1.2)	8	6	−2	9	Negative Coombs' serum, rise in lactic dehydrogenase, 168–297 IU/ 100 ml
3	52	4.6	4	9	−2	4	Normal haptoglobin, negative Coombs' serum
4	22	2.4	8	7	−2	8	Serum hemoglobin and haptoglobin normal, negative Coombs' serum
5	24	1.7 (D-0.7)	7	2	−1	7	Negative Coombs' serum, normal haptoglobin
6	24	1.6	6	2	−1	6	Plasma hemoglobin, 31 mg/100 ml, negative Coombs' serum
7	24	2.6	7	4	−2	10	Negative Coombs' serum, haptoglobin fell from 224 to 137 mg/ 100 ml on Day 7

*Liver function studies normal in all patients.
†Indicates amount of direct-reacting bilirubin.

ized coronary atherosclerosis, occlusion of the anterior descending coronary artery, and ventricular aneurysm.

There was only one instance of bleomycin pneumonitis (table 4, patient No. 12). Death resulted directly from pulmonary insufficiency, at a total bleomycin dose of 600 mg. Widespread seminoma was under partial control at the time of death.

DISCUSSION

The half-life of bleomycin in the blood is short, probably less than 2 hours, and tissue inactivation is rapid, especially by the liver and

kidney (4). Since bleomycin affects cells in all phases of the mitotic cycle but especially in the M and G_2 phases (5), it is logical to administer the drug by continuous infusion for at least 4–5 days assuming the Tc of the neoplastic germinal cell approaches that of other mammalian tumor cells. Cvitkovic et al (6) have recently reported on continuous bleomycin infusion alone in squamous cell carcinoma of the cervix and head and neck areas. They noted a 53% response rate in a small series of 17 patients. In a previous report (7), using an intermittent bleomycin schedule, remissions were obtained in only six of 64 patients (9%) who had cervix and head and neck cancer.

The overall response rate of 73% achieved with the present study is the same as that achieved with our first bleomycin program. Further, the complete response rate of 40% is not impressively better than the 32% complete response rate obtained with intermittent bleomycin schedules. However, almost all of our patients are in the advanced-disease category implying large bulky masses. Previously reported data (2) show a very low complete response rate of 12% for patients with advanced abdominal disease and 19% for patients with advanced pulmonary disease. With our program, all but one of the complete responses occurred in patients with advanced disease. Hence, the continuous infusion protocol appears to be superior for this subclass of stage III neoplasia.

Toxicity in terms of leukopenia and thrombopenia is no more severe than that encountered with the intermittent bleomycin programs. However, there is the added feature of hyperbilirubinemia which may be of hemolytic origin but is brief and does not require transfusion. In experimental animals, bleomycin has been found capable of producing liver toxicity (8,9). Our data do not as yet suggest this possibility and unfortunately, a liver biopsy cannot be performed safely immediately after chemotherapy.

Hypertension was a new and unexpected complication and probably directly contributed to the death of one patient from acute myocardial infarction. At age 52 this patient was considered elderly for the group and a postmortem examination showed extensive substrate coronary atherosclerosis.

Bleomycin pneumonitis remains a serious threat. However, this was encountered only once, resulting in the patient's death despite corticosteroid treatment. Pulmonary fibrosis does not appear to be increased by continuous bleomycin infusion.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Bleomycin: NSC-125066; Bleo
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-
Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1); Oncovin

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Unorthodox Therapy for Murine Neuroblastoma With 6-Hydroxydopamine (NSC-233898), Bretylium Tosylate (NSC-62164), Papaverine (NSC-35443), and Butyric Acid (NSC-8415) [1,2,3]

Brief Reports and Preliminary Communications

Jerry Z. Finklestein, Karen Tittle, Rosalie Meshnik, and John Weiner [4,5,6]

The effects of 6-hydroxydopamine, bretylium tosylate, papaverine, and butyric acid were evaluated in A/J mice with palpable neuroblastoma. These studies were performed because of reports that 6-hydroxydopamine (1,2) and bretylium tosylate (2) may cause selective destruction of sympathetic nerve endings in vivo. Papaverine has been reported to cause murine neuroblastoma cells to differentiate in vitro (3) and butyric acid inhibits cell division of murine neuroblastoma cells in culture (4).

METHODS

The murine model has been described previously (5), and thus the therapeutic aspects of the system will be briefly summarized. Adult A/J mice obtained from Jackson Laboratories, Bar Harbor, Me, were quarantined for 2 weeks and then inoculated subcutaneously (sc) in the interscapular area with 10^6 neuroblastoma tumor cells. Toxicity studies were performed with 6-hydroxydopamine, bretylium tosylate, papaverine, and butyric acid. All agents were diluted with saline alone except papaverine, which was suspended in saline plus one of the following: Tween 80 (Sigma Chemical Co.), acacia (B.R. Elk & Co.), or carboxymethyl cellulose (Sigma Chemical Co.), with or without ultrasonification. All drugs were administered intraperitoneally (ip) to randomized groups of normal mice.

The therapeutic studies were conducted using nonlethal, highest nontoxic, and lowest toxic doses of drug. These drugs were administered when the sc implanted interscapular tumor measured 1.0–1.5 cm in diameter. Additional experiments were performed with 6-hydroxydopamine. In one series of experiments, the drug was injected 30 minutes after inoculation of tumor cells while in another series of experiments it was injected directly into the tumor in vivo. Control mice received normal saline in the appropriate fashion.

RESULTS

Table 1 outlines the drug toxicity studies conducted in adult A/J mice who received various doses of the agents. It was not possible to formulate an injectable suspension with papaverine using dosages greater than 5 mg/10 g of body weight. The effects of chemotherapy on the growth of the sc implanted neuroblastoma are tabulated in table 2. All four agents were ineffective in the dosages and regimens used. Table 2 also includes results previously published

[1] Received July 15, 1974; revised Dec 27, 1974; accepted Jan 22, 1975.

[2] Supported in part by Public Health Service grants CA-14658, CA-14560, and CA-14089 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[3] 6-Hydroxydopamine: 1,2,4-benzenetriol, 5-(2-aminoethyl)-, hydrochloride. Supplied by Sigma Chemical Co., St. Louis, Mo.

Bretylium tosylate: CAS reg. No. 61-75-6; ammonium, (*o*-bromobenzyl)ethyldimethyl— *p*-toluenesulfonate. Supplied by Dr. R. A. Maxwell, The Wellcome Research Laboratories, Triangle Park, NC.

Papaverine: CAS reg. No. 61-25-6; isoquinoline, 6,7-dimethoxy-1-veratryl-, hydrochloride. Supplied by Sigma Chemical Co., St. Louis, Mo.

Butyric acid: CAS reg. No. 107-92-6; sodium butyrate. Supplied by MC/B Chemists, Norwood, Ohio.

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[5] The authors wish to thank Dr. John E. Byfield for his helpful advice and suggestions.

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TABLE 1.—Toxicity studies of 6-hydroxydopamine, bretylium tosylate, papaverine, and butyric acid in normal adult A/J mice*

Drug	Schedule of administration	Dose (mg/10 g body weight)	No. of mice dying/No. of mice treated	Mean time (days) to death
6-Hydroxydopamine	Days 1–6, 8–13, 15–20	0.5	0/6	—
		1.0	1/8	19
		1.5	5/5	9
		2.0	3/3	7.3
Bretylium tosylate	Days 1–5, 8–12, 15–19	0.1	0/6	—
		0.2	0/9	—
		0.3	1/9	11.0
		0.4	9/9	11.0
		0.5	3/3	2.5
Papaverine	Days 1–5, 8–12	3.0	0/9	—
		5.0	0/3	—
Butyric acid	Days 1–5	5.0	0/5	—
		10.0	0/4	—
		20.0	0/8	—
		30.0	0/3	—
		40.0	6/9	6.3
		50.0	6/6	2

*All animals were observed for a minimum of 30 days after injections.

(5) for two active agents—cyclophosphamide and BCNU.[†]

DISCUSSION

6-Hydroxydopamine and bretylium tosylate offer the chemotherapist compounds which may have the ability to destroy sympathetic nerve endings. 6-Hydroxydopamine was used in the model because it has been shown that it can cause morphologic alterations of adrenergic nerve terminals (2,6,7). It has the potential ability to create a chemical sympathectomy. In addition, it is active against a newborn's neurons, and could reasonably be expected to have some activity in an embryologic neural tumor with sparing of adult mature ganglion and nerve endings. Injections of bretylium tosylate in newborn mice and rats during the first week of life result in a considerable decrease in the size of the sympathetic chain ganglia (7).

Papaverine was used because it has been shown to induce morphologic differentiation of murine neuroblastoma cells in vitro. Prasad et al have shown that these agents must be present to demonstrate morphologic differentiation, and that upon removal and subsequent addition of fresh growth media all in vitro changes are reversed (3). Although the cellular factors which regulate the expression of differentiated functions in neuroblastoma in vitro are not well understood, cyclic adenosine monophosphate (AMP) does induce differentiated functions and both papaverine and butyric acid have been hypothesized to elevate cyclic AMP levels (3,4).

Our results suggest that 6-hydroxydopamine does not alter the course of progressive C1300 murine neuroblastoma. This is in keeping with the report of Rossier et al, who believe that 6-hydroxydopamine has a nonspecific effect, if any, on the murine neoplasm (8). Although papaverine has had a limited trial as an antitumor agent in children (9), alkylating agents such as cyclophosphamide and BCNU appear to be more promising at this time (table 2).

Pediatric oncologists have been frustrated by the lack of efficacious chemotherapeutic regi-

[†] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

TABLE 2.—Effect of drugs on neuroblastoma in adult A/J mice with sc implanted tumors*

Drug	Ip dose (mg/10 g)	Dose schedule	No. of mice	Survival time (days)		
				Range	Median	ILS (%)†
6-Hydroxy-dopamine	Control		29	4–26	10	—
	0.5	Days 1–6, 8–13, 15–16	15	3–16	14	<50
	0.5	Every other day	10	5–15	8	<50
	0.75	Days 1–6, 8–13	10	6–9	8	<50
	1.0	Days 1–6, 8–13	10	6–9	8	<50
6-Hydroxy-dopamine	Control in tumor‡		10	8–12	8	—
	0.5	Days 1–6, 8–13, 15–17 in tumor‡	10	6–17	9	<50
6-Hydroxy-dopamine	Control + tumor§		20	14–39	21	—
	0.5	Days 1–6, 8–13, 15–19, 21–25 + tumor§	10	18–25	21	<50
	0.75	Days 1–6, 8–13, 15–19, 21–25	10	19–33	22	<50
	1.0	Days 1–6, 8–13, 15–19, 21–22	9	15–22	22	<50
Bretylium tosylate	Control		15	4–13	9	—
	0.1	Days 1–5, 8–12, 15–19	10	9–18	12.5	<50
	0.2	Days 1–5, 8–12, 15–19	10	7–25	9	<50
	0.3	Days 1–5, 8–12, 15–19	10	4–12	6.5	<50
	0.4	Days 1–5	10	2–8	2.5	<50
Papaverine	Control		8	4–15	12	—
	1.0	Days 1–5, 8–10	10	3–10	7	<50
	5.0	Days 1–5, 8–12	9	5–12	8	<50
Butyric acid	Control		30	4–15	9.5	—
	10	qd × 5	10	5–12	8	<50
	20	qd × 5	10	3–23	10	<50
	30	qd × 5	10	3–10	3	<50
	40	qd × 5	10	2–8	3	<50
BCNU	Control		13	7–24	10	—
	0.18	× 1	13	13–29	20	100¶
	0.24	× 1	13	13–84	20	100¶
Cyclophos-phamide	Control		16	4–16	9	—
	2.0	× 1	17	7–27	16	77¶
	2.5	× 1	17	21–38	27	200¶

*Control mice received 0.2 cc of normal saline.

†% ILS = percent increase in lifespan of treated over control animals.

‡6-Hydroxydopamine or normal saline (control) was inoculated directly into the tumor.

§Tumor cells and drug were administered on the same day; all other chemotherapy experiments were conducted on animals with palpable tumors of 1.0–1.5 cm in diameter.

¶Values for BCNU were significant at $P = 0.016$; values for cyclophosphamide were significant at $P < 0.00001$ for the 2.5 mg/10 g dose and $P = 0.006$ for the 2.0 mg/10 g dose. These values were analyzed by the method for exact probabilities for contingency tables described in ref 10.

mens for the child with widespread stage IV neuroblastoma. The murine neuroblastoma model is sensitive to cyclophosphamide, a drug known to be active in childhood neuroblastoma (5). If the C1300 murine model is relevant to the human disease, then the screening experiments performed with the four unorthodox therapeutic agents described here suggest that these therapies will be ineffective for children with widespread metastatic neuroblastoma.

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Absorption of Leucovorin (NSC-3590) From a "Mouthwash" [1,2,3]

Brief Reports and Preliminary Communications

Howard W. Bruckner [4] and Joseph R. Bertino [5,6]

Leucovorin, a reduced folate, reverses the effects of methotrexate (1,2).[7] The ratio of methotrexate to leucovorin, the time of exposure to each, and the route of administration may sometimes determine the effectiveness of leucovorin and the therapeutic index of methotrexate (1,2). Stovner et al (3) reported that mucositis due to chemotherapy with methotrexate could be prevented by using leucovorin with proteolytic enzymes in a mouthwash.

In this communication we report that a small amount of leucovorin is absorbed when used as a mouthwash; however, the serum levels of leucovorin should be insufficient to antidote the systemic therapeutic effects of high-dose methotrexate. Thus, this approach to prevent mucositis after methotrexate therapy should be explored further.

MATERIALS AND METHODS

Men who had volunteered to be controls rinsed their mouths for 1 minute with 3 mg of commercially available leucovorin diluted to 10 ml with 0.9% NaCl. The mouthwash was expectorated after 1 minute and the participants expectorated all saliva during the next 10 minutes, finally rinsing their mouths with 30 ml of normal saline. Fasting-serum specimens obtained at 0, 30, 60, and 120 minutes were measured for available serum folate with a *Lactobacillus casei* bioassay (4).

RESULTS

Serum folates increased 50%–300% as shown in table 1. Increments in available serum folate ranged from 4.6 to 14 ng/ml.

DISCUSSION

Mucositis is a common and distressing side effect of methotrexate therapy, frequently leading to delays in therapy and poor patient acceptance. A simple, effective method of obviating this oral ulceration without compromising the therapeutic effect of methotrexate would be most worthwhile. A previous study reported that leucovorin, used with hyaluronidase, appeared to decrease the severity of mucositis produced by methotrexate given intra-arterially. The present study was initiated to determine if leucovorin administered as a mouthwash without hyaluronidase could be absorbed and could enter the systemic circulation. The absorption of leucovorin may have clinical importance regardless of the mechanism of absorption. Small amounts of leucovorin may quantitatively affect the toxic or therapeutic potency of methotrexate.

Serum folate levels were found to increase after small (3 mg) single doses of leucovorin. The levels produced (10^{-8} M) would not be expected to reverse the therapeutic effects of methotrexate given in pulse doses of 25 mg or greater (1, 2, 5).

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[2] Supported by Public Health Service Special Fellowship IF-3 and grant CA-51650 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare; and American Cancer Society grant PF 826.

[3] Leucovorin: CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt; 5-formyltetrahydrofolate.

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[7] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-.

TABLE 1.—Available serum folate before and after the administration of a leucovorin mouthwash*

Participant	Serum folate (ng/ml) obtained at— (*min*)			
	0	30	60	120
A	4.8	16.5	17.0	14.5
B	5.2	5.6	9.2	13.0
C	4.4	8.0	—	9.0
D	20.0	34.0	25.0	28.0

*L. *casei* assay; mouthwash = 3 mg of leucovorin in 10 ml of 0.9% saline.

The mechanism of absorption is not entirely clear. While direct absorption through oral mucosa is the most likely explanation, inadvertent swallowing of material could also have occurred. Previous evidence from this laboratory has shown that leucovorin administered orally is absorbed well and enlarges the reduced folate pool (6). As little as 0.15 mg of leucovorin, given parenterally, can produce a 10^{-8} M level of reduced folate (6).

The appearance of statistically significant elevations of available folate in serum after only 30 minutes in three of four participants suggests direct absorption from the oral mucosa. The later and continued increase in the case of participant B (table 1) can be explained by inadvertent swallowing. The high baseline level (20 ng/ml) of available folate is unexplained in participant D; nevertheless, available folate increased within 30 minutes of applying the mouthwash.

Two possible mechanisms could explain why the increased folate levels obtained by a single mouthwash may prevent excessive side effects of methotrexate: (*a*) direct entry of high concentrations of leucovorin into the cells of the oral mucosa, and (*b*) termination of bone marrow toxicity during the third phase of methotrexate excretion when serum concentrations are as low as 10^{-8} M. The appearance of 10^{-8} M increments in the levels of reduced folate after only 30 minutes requires rapid absorption of at least 5% of the leucovorin (0.15 ng/3 mg) and does not take into account a potentially substantial additional amount which may be localized in cells along the route of absorption. The data suggest a careful therapeutic trial of leucovorin as a "mouthwash" to prevent oral ulceration, especially with the large intermittent doses of methotrexate, with and without leucovorin, currently in use.

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Phase II Study of Chromomycin A_3 (NSC-58514) in Advanced Colorectal Carcinoma [1,2,3]

C. G. Moertel, A. J. Schutt, R. G. Hahn, T. A. Marciniak, and R. J. Reitemeier [4,5]

Brief Reports and Preliminary Communications

Chromomycin A_3 is an antibiotic isolated from cultures of *Streptomyces griseus* which has been shown to have antineoplastic activity in several model tumor systems (1–3). In clinical trials in Japan and South Africa, objective responses were obtained in patients with lymphomas, sarcomas, and gastric and colorectal carcinomas (4,5). In a previous phase I study of chromomycin A_3 given in a 5-day intensive course (6), we found only minimal hematologic effects and the dose-limiting toxicity was acute renal tubular injury manifested by proteinuria and azotemia. The maximum clinically tolerable dose was 0.9 mg/m²/day given for 5 days.

The purpose of this study was to determine the therapeutic activity in advanced colorectal carcinoma of chromomycin A_3 administered in a 5-day intensive course at a maximum tolerable dosage.

MATERIALS AND METHODS

Patients

Twenty-seven patients were chosen for this study. All patients had histologically confirmed adenocarcinomas which originated in the colon or rectum and a measurable area of known malignant disease which served as an objective indicator of response to therapy. Each patient was ambulatory and was nutritionally stable prior to initiation of treatment. Treatment was deferred for at least 1 month after any previous chemotherapy, radiation therapy, or abdominal surgery which involved a resection or anastomosis and for at least 3 weeks after abdominal surgery which involved exploration and biopsy only. Significant renal disease, defined as a serum creatinine of 1.5 mg/100 ml or greater, was an absolute contraindication to therapy. To insure the optimum conditions for demonstrating therapeutic activity, particular emphasis was placed upon selecting patients who were in good general condition and securing a significant representation of patients who had not been previously exposed to cytotoxic drugs.

Drug Administration

Chromomycin A_3 was dissolved in sterile water at a concentration of 0.1 mg/ml and given immediately by rapid intravenous push into the tubing of an established infusion of 5% dextrose in water. The planned course of therapy was 0.9 mg/m²/day for 5 consecutive days. Phenothiazine antiemetics and parenteral hydration were employed if the patient experienced significant nausea and vomiting.

Prior to therapy, each patient had a general medical evaluation including the following laboratory studies: urinalysis, hemoglobin level, leukocyte and platelet counts, serum creatinine, calcium, bilirubin, alkaline phosphatase, glutamic oxaloacetic transaminase, sodium potassium, uric acid, and glucose, as well as a chest roentgenogram and roentgenograms of any osseous lesions. Leukocyte and platelet counts and serum creatinines were repeated on Day 5 of treatment. After treatment, leukocyte counts were obtained twice weekly and platelet counts once weekly. Patients were reevaluated at 5 weeks with repetition of all pretreatment laboratory studies. Patients who remained in good condition and showed no evidence of ob-

[1] Received Dec 17, 1974; accepted Jan 22, 1975.

[2] Supported by contract NO1-CM-02066 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Chromomycin A_3: CAS reg. No. 7059-24-7; Toyomycin. Supplied by Cancer Therapy Evaluation, DCT, NCI.

[4] Mayo Clinic and Mayo Foundation, Rochester, Minn.

[5] *Reprint requests to:* Charles G. Moertel, MD, Division of Gastrointestinal Oncology, Mayo Clinic, Rochester, Minn 55901.

jective tumor progression were re-treated every 5 weeks until tumor progression was evident.

RESULTS

The characteristics of the patients chosen for study are shown in table 1. All of these patients were ambulatory, most of them were capable of working full- or part-time, and the majority had not had previous chemotherapy.

Twenty patients had the entire planned initial course of therapy. Seven patients did not have their planned fifth dose of therapy because of a rising creatinine that was observed on Day 5.

Toxicity

The toxic reactions experienced by our patients are shown in table 2. They were comparable in nature and severity to those which we observed in our phase I study. Nausea and vomiting were frequent, troublesome, and incompletely relieved by antiemetic drugs. Although vomiting was usually limited to the treatment period, nausea and anorexia frequently persisted for several days after therapy. Hematologic toxic reactions were limited to four patients who had thrombocytopenia with nadirs of 131,000, 121,000, 61,000, and 34,000 cells/mm^3. Twelve patients had evidence of nephrotoxic effects consisting of proteinuria and significant elevations of serum creatinines. These were usually completely reversible, although four patients had continued mild creatinine elevations at 5 weeks. In one patient, acute renal injury probably contributed to his death. This patient had a complete small bowel obstruction secondary to carcinoma and coincident with the completion of therapy. In spite of adequate parenteral hydration, his serum creatinine increased to 12 mg/100 ml. With management of his renal failure, he eventually had diureses and the creatinine decreased to 7 mg/100 ml by the day preceding his death (25 days after initiation of chromomycin A_3 therapy).

Therapeutic Results

After 5 weeks of treatment, 21 patients showed objective progression of their malignant disease and were not re-treated. One patient whose disease was objectively stable was not re-treated because of a persistent elevation in serum creatinine. Four patients whose diseases were stable at 5 weeks were re-treated but objective progression of disease was seen at 10 weeks. One patient's disease remained objectively stable at 5 and 10 weeks but showed progression at 15 weeks and after three courses of therapy. Only two patients claimed symptomatic improvement and only one had transient improvement in performance score.

TABLE 1.—Characteristics of 27 patients treated with chromomycin A_3

Patient characteristics	No. of patients
Sex (M/F)	13/14
Age (yrs)	
Mean = 63	
Range = 36–77	
Performance score*	
0–1	18
2–3	9
Chemotherapy	
Prior	12
None	15
Prior radiation therapy	2

*Based on Eastern Cooperative Oncology Group scale of 0 (fully active) to 4 (totally disabled).

TABLE 2.—Toxic reactions of 27 patients

Toxic reactions	No. of patients
Nausea	25
Vomiting	21
Leukopenia	0
Thrombocytopenia (cells/mm^3)	
<150,000 >100,000	2
<100,000	2
Creatinine rise (mg/100 ml)	
>1.3 <2.0	2
>2.0 <3.0	4
>3.0	6

DISCUSSION

In this phase II study of chromomycin A_3 administered by a 5-day intensive course for advanced colorectal carcinoma, the principal side effects were nausea, vomiting, proteinuria, and azotemia. No evidence of therapeutic ac-

tivity was observed among 27 patients who were treated. We must emphasize, however, that both the toxic reactions and the therapeutic results recorded here can only be related to the intensive course schedule of administration which we employed. The favorable results reported in overseas studies were obtained by delivering smaller doses of chromomycin A_3 repeatedly over a much more prolonged time period. Final judgment regarding the therapeutic potential of chromomycin A_3 must be reserved until other dosage schedules have been explored.

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Phase II Study of Cytembena (NSC-104801) in Advanced Colorectal Carcinoma [1,2,3]

C. G. Moertel, A. J. Schutt, R. G. Hahn, T. A. Marciniak, and R. J. Reitemeier [4,5]

Brief Reports and Preliminary Communications

Cytembena is an original Czechoslovakian cytotoxic drug prepared by hydrolysis of antineoplastically active crotonolactones. In animal tumor screens it has been shown to have activity against Sarcoma 180, adenocarcinoma of the lactic gland in mice, Ehrlich ascitic carcinoma, Yoshida ascitic sarcoma, and Zojdeln hepatoma in rats (1,2). The drug, however, is not active against the L1210 leukemia system.

Pharmacokinetic studies of cytembena in man conducted by Grafnetterová et al (3) showed a remarkably long serum half-life. It seems to be avidly absorbed by the renal tubules and the primary route of elimination is thought to be extrarenal, probably hepatic. Early clinical trials with cytembena conducted in Czechoslovakia have been reported to show evidence of significant human antineoplastic activity particularly in gynecologic neoplasms (4).

In a phase I study of cytembena administered by a 5-day intensive course,[6] we found the dose-limiting toxicity to be an "autonomic storm" phenomenon consisting of hypertension, tachycardia, tachypnea, hyperperistalsis, frequent explosive defecation, facial flushing, paresthesia, and chest pain with accompanying ischemic electrocardiographic changes. We found no evidence of hematologic toxic effects. Based on our phase I study, we felt that a regimen of 200 mg/m² given twice daily over 5 consecutive days (total dose, 2000 mg/m²) was clinically tolerable in patients without prior coronary artery disease or impaired renal function.

No prior experience with cytembena therapy for large bowel carcinoma has been recorded. The purpose of this study was to determine the therapeutic activity of cytembena in one of the "signal" tumors, advanced colorectal cancer, by administering the drug in a 5-day intensive course at a maximum clinically tolerable dosage.

MATERIALS AND METHODS

Patients

Twenty-six patients were chosen for the study. All had histologically confirmed adenocarcinomas originating in the colon or rectum and all had a measurable area of known malignant disease to serve as an objective indicator of response to therapy. Each patient was ambulatory and maintaining a reasonable state of nutrition before the start of treatment. Treatment was deferred for at least 1 month after any previous chemotherapy, radiation therapy, or abdominal surgery involving a resection or anastomosis. It was deferred for at least 3 weeks after abdominal surgery involving exploration and biopsy only. Significant renal disease, defined as a serum creatinine level of 1.5 mg or greater, was an absolute contraindication to therapy. Any evidence of coronary artery disease, eg, past history, current symptoms, or electrocardiographic abnormalities,

[1] Received Dec 17, 1974; revised Jan 30, 1975; accepted Feb 21, 1975.

[2] Cytembena: CAS reg. No. 16170-75-5; acrylic acid, 3-*p*-anisoyl-3-bromo-, sodium salt; sodium *B*-4-methoxybensoyl *B-cis* bromoacrylate. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

[3] Supported by Public Health Service contract CM-02066 from the DCT, NCI, National Institutes of Health, Department of Health, Education, and Welfare.

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[5] *Reprint requests to:* Charles G. Moertel, MD, Gastrointestinal Oncology, Mayo Clinic, Rochester, Minn 55901.

[6] Frytak S, Moertel CG, Schutt AJ, et al. A phase I study of cytembena. Cancer. In press.

was also an absolute contraindication to therapy. To insure optimum conditions for demonstrating therapeutic activity, particular emphasis was placed on selecting patients in good general condition and in securing a significant number of patients who had not had previous exposure to cytotoxic drugs.

Administration of Drug

Cytembena was dissolved in 10 cc of sterile water and slowly injected into the side arm of a freely running intravenous infusion. The planned dose was 200 mg/m² twice daily for 5 consecutive days. Pulse, temperature, and blood pressure were determined prior to drug administration and at 5- and 15-minute intervals after administration. Phenothiazine antiemetics and parenteral hydration were employed if the patient experienced significant nausea and vomiting.

Prior to therapy each patient had a general medical evaluation including laboratory studies (urinalysis, hemoglobin, leukocyte count, platelet count, serum creatinine, calcium, bilirubin, alkaline phosphatase, glutamic oxaloacetic transaminase, sodium, potassium, uric acid, and glucose), an electrocardiogram, a chest roentgenogram, and roentgenograms of any osseous lesions. An electrocardiogram and a serum creatinine level were also obtained on Day 5. Patients were re-evaluated at 5 weeks with repetition of all pre-treatment laboratory studies. Patients who remained in good clinical condition and showed no evidence of objective progression were re-treated every 5 weeks until progression was evident.

RESULTS

The characteristics of the patients chosen for the study are shown in table 1. All were ambulatory, most were capable of working full- or part-time, and 12 of the 26 had had no previous chemotherapy.

One patient who had jaundice before treatment showed significant clinical deterioration with signs of portal systemic encephalopathy on Day 2 of treatment. In view of these changes, it was elected not to complete his planned therapy. We did not feel that the single day of cytembena therapy had contributed in any significant degree to the patient's deterioration. The observations to follow will include only those remaining 25 patients who completed their entire planned course of treatment.

Toxicity

The toxic reactions experienced by our patients are shown in table 2. They were com-

TABLE 1.—Characteristics of patients treated with cytembena

Patient characteristics	No. of patients
Sex (M/F)	12/14
Age (yrs)	
Mean = 56	
Range = 37–73	
Performance score*	
0–1	15
2–3	10
Chemotherapy	
Prior	14
None	12
Prior radiation therapy	4

*Based on Eastern Cooperative Oncology Group scale of 0 (fully active) to 4 (totally disabled).

TABLE 2.—Toxicity in 25 patients treated with cytembena

Toxic reaction	No. of patients
Nausea	23
Vomiting	17
Polyuria	3
Facial paresthesia	4
Jaw pain	2
Arm pain	14
Chest pain	6
Increased systolic blood pressure (>20 mm Hg)	8
Increased diastolic blood pressure (>20 mm Hg)	6
Electrocardiographic change	8
Creatinine rise	
>1.3 mg <2.0 mg	6
>2.0 mg	2

parable in nature and severity to those we observed at similar dosage levels in our phase I study.

Transient arm pain coincident with injection and persistent for a few minutes thereafter was experienced by most patients. Less frequently, this effect was accompanied by chest pain, jaw pain, and facial paresthesia. Although a few patients had nausea and vomiting on Day 1, most did not experience these symptoms until Day 3, consistent with an accumulative effect of the drug. Vomiting could usually be aided by antiemetics, but in one patient who also had significant impairment of liver function, vomiting was severe and intractable. Significant elevation in blood pressure was also usually delayed until Day 3 or 4. Electrocardiographic changes were limited to T wave inversions in six patients, but two also had significant depression of the S-T segments. There was a coincidence of cardiovascular side effects, with six of the eight patients with electrocardiographic changes also having significant blood pressure elevation. Elevations of serum creatinine were noted in eight patients on Day 5, but only two were over 2.0 mg. In all patients, the changes in blood pressure, electrocardiogram, or creatinine had reverted to normal at the time of their 5-week evaluation. In no instance were any of these side effects lethal or life threatening.

Therapeutic Results

Among the 25 patients treated with a full intensive course of cytembena, 18 showed objective progression at 5 weeks and were not re-treated. Four were stable at 5 weeks, but showed progression at 10 weeks and after two courses of therapy. The remaining three were stable at 5 and 10 weeks but showed progression at 15 weeks. Six patients claimed symptomatic improvement 5 weeks after their first course of therapy. Only one had improvement in performance score.

DISCUSSION

In this phase II study of cytembena administered by a 5-day intensive course, the primary side effects were nausea, vomiting, arm pain, chest pain, facial paresthesia, transient hypertension, transient electrocardiographic changes, and transient elevation in serum creatinine levels. These effects duplicate our phase I experience, indicating that the dose-limiting toxicity of cytembena by this schedule is mediated through the autonomic nervous system.

No evidence of therapeutic activity was seen among our 25 adequately treated patients with colorectal carcinoma. We must conclude that cytembena given by the method we employed has no role in the chemotherapy of colorectal carcinoma. We must emphasize, however, that both the toxic reactions and the therapeutic results recorded here can only be related to the particular type of neoplasm treated and to the intensive course schedule we employed. The favorable results obtained by Czechoslovakian workers were primarily in gynecologic cancers, and these investigators delivered cytembena in smaller doses over a much more prolonged time period. Final judgement regarding the therapeutic potential of cytembena must be reserved until other dosage schedules have been explored and until phase II studies have been conducted in a broader spectrum of neoplasms.

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Antitumor Activity in *Pimelea simplex* [1]

H. T. C. Howard and M. E. H. Howden [2,3,4]

Pimelea simplex (desert rice flower) is a small grassy plant that occurs widely in the eastern states of Australia. It is a member of the family Thymeleaceae. It has been implicated as a cause of St. George disease in cattle (1). The symptoms of this disease are: gastrointestinal disturbance with acute diarrhea; heart, lung, and liver changes including severe subcutaneous ventral edema; and a fall in the erythrocyte series parameters. Cashmore (2) observed similar symptoms in sheep and cattle in New Zealand which were grazed on paddocks containing a related species, *Pimelea prostrata*. He identified prostratin, the 13-acetate of 12-desoxyphorbol, as the primary cause of the symptoms. Phorbols are a class of irritant compounds exhibiting cocarcinogenic properties (3). They occur for example in *Croton tiglium* L. (4). Other thymeleaceous species, for example *Daphne mezereum* L., have been found to contain compounds such as daphnetoxin (5) which has strong irritant effects.

Since 1973 we have been working on the isolation and identification of the toxin(s) in *Pimelea simplex* affecting cattle. We now report the first observations of antitumor activity in this plant. *Pimelea simplex* was collected in the West Darling region of New South Wales in March of 1973. Whole plants were dried in a hot air oven at 43°C and then milled to pass through a 1-mm mesh. The milled plant (60 g) was extracted with ethanol in a Soxhlet extractor over a 2-day period. The extract was evaporated in vacuo at 50°C and then dried in a vacuum desiccator. The dark-green sticky extract (5.0 g) was then screened by the NCI for antineoplastic activity.[5] The results are summarized in table 1 and indicate activity in the P388 mouse leukemia test system. A second sample was prepared in a similar manner and table 1 shows that the activity was confirmed. Dr. J. L. Hartwell informed us that the observed high antitumor activity at such low dosage levels for an initial crude extract is rare.[6]

We are currently investigating whether the cattle toxin(s) in *Pimelea simplex* is the source of its antineoplastic activity. If a phorbol-like toxin(s) is present in *Pimelea simplex*, as seems probable, then cocarcinogenic properties (3) might be expected for them. A link between the toxin(s) and anticancer activity would provide an example of "Haddow's paradox" (6). We are in the process of isolating and identifying the antitumor agents in *Pimelea simplex* and are screening all other *Pimelea* species occurring in Australia for antitumor activity.

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[1] Received Dec 17, 1974; revised Feb 13, 1975; accepted Feb 19, 1975.

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[3] The authors wish to thank Dr. J. L. Hartwell of Drug Research and Development (DRD), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health (NIH) for testing the extracts; Dr. A. Charlson, Associate Prof. T. J. McClure, and Mr. H. B. Roberts for valuable advice and encouragement; and Messrs. J. Bridges, M. Bye, and M. Tully for generous assistance in collection of the plant material.

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[5] Full details of the anticancer screening may be obtained from DRD, DCT, NCI, NIH, Bethesda, Md, by referring to NSC-B664027.

[6] Hartwell JL. Personal communication. July 10, 1974.

TABLE 1.—Antitumor activity*

Sample No.	Dose/ injection (mg/kg)	No. of survivors/ No. of animals tested†	Average control weight change (g)	Mice weight difference: T-C‡ (g)	Tumor evaluation—median survival time (days)		T/C (%)
					T	C	
1	400	0/3	1.1	−1.1	2.0	11.0	
	200	0/3	1.1	−1.1	2.0	11.0	
	100	0/3	1.1	−1.1	3.0	11.0	
	50.0	2/3	1.5	−5.3	7.0	11.0	
	25.0	3/3	1.5	−4.6	21.0	11.0	190
	12.5	3/3	1.5	−6.5	17.0	11.0	154
2	37.5	1/3	0.7	−6.0	4.5	10.8	
	25.0	3/3	0.7	−5.7	20.5	10.8	189
	16.5	3/3	0.7	−5.4	18.0	10.8	166
	11.0	3/3	0.7	−5.0	17.5	10.8	162
	84.3	1/6	1.2	−7.0	3.8	12.2	
	56.2	6/6	1.2	−5.7	19.3	12.2	158
	37.5	5/6	1.2	−4.6	18.8	12.2	154
	25.0	6/6	1.2	−4.1	17.1	12.2	140
	16.5	6/6	1.2	−3.6	18.8	12.2	154
	11.0	6/6	1.2	−2.6	16.3	12.2	133

*Test system was P388 lymphocytic leukemia for mice; activity was measured by median survival time; inoculum was injected ip in ascitic fluid at a level of 10^6 cells. For each test there were 9 ip injections at daily intervals of extract in hydroxypropylcellulose medium; results were evaluated on Day 30 of the test.

Solutions of the extract in saline were tested by cell culture tube assay on human epidermoid carcinoma of the nasopharynx and found to be inactive; the ED50 was found to be more than 100 μg/ml, and the slope, or change of response for each 1-log change of dose, was 0.

†Recorded on Day 5 as a measure of extract toxicity.

‡Average weight change of test (T) group minus average change of control (C) group.

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The Role of Metal Complexes and Metal Salts in Cancer Chemotherapy

A Symposium Presented at the 30th Southwest Regional Meeting of the American Chemical Society

Houston, Texas
December 10–11, 1974

The nine papers presented at the symposium on The Role of Metal Complexes and Metal Salts in Cancer Chemotherapy report on the current clinical status of platinum complexes, recent biochemical studies involving a variety of platinum compounds, and the anti-tumor activity of rhodium(II) carboxylates and group IIIa metal salts.

I wish to express my appreciation to A. P. Kimball for his extensive assistance in organizing the Symposium, and to the authors for making the symposium a success.

John L. Bear

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Possible Mechanisms for the Antitumor Activity of Platinum Coordination Complexes[1]

Barnett Rosenberg [2,3]

There is now compelling evidence that coordination complexes of the platinum-group metals are a new class of potent antitumor agents. While the sophistication of this area of chemistry has made gargantuan strides over the past 40 years, the pharmacologic investigation and uses of metal complexes in medicine have fallen into a state of benign neglect. Bioinorganic chemistry, a new field, is now rectifying this defect. One small example is described here.

Five years ago, our group discovered the antitumor activity of *cis*-dichlorodiammineplatinum(II),[4] the classic Peyrones' chloride, and a number of its analog structures. Succeeding work by many laboratories has advanced the development of this first drug to the point where it is now in clinical trials in man as an anticancer agent. The results of these trials are discussed in some detail by Gottlieb et al (1) and Hill et al (2) and I will defer to their expertise. Similarly, the molecular biology of the action of the drugs, particularly with regard to DNA, is reported by Drs. Munchausen and Rahn (3), and I will comment only passingly on this aspect. In recent years, there have been a number of review papers covering all aspects of developments in this field (4–9), and I do not propose to repeat any of these reviews. Instead, I should like to discuss some ideas that have evolved in our laboratory with regard to the systemic mechanisms of action of this class of drugs, and some of the recent evidence consistent with, but unfortunately not corroborative of, these ideas. To do so, I must review briefly some of the salient features of the chemistry and biologic actions of these drugs.

Chemistry of Square Planar Platinum (II) Complexes

Since *cis*-dichlorodiammineplatinum(II) was one of the first metal complexes shown to be active and was chosen by the National Cancer Institute as the first for clinical trials, it has been imbued with primacy and, perhaps, undue importance. At this meeting we have discussed a large number of coordination complexes which, to the extent that they exhibit similar biologic activities, may be considered a unified class of potent drugs. In many laboratories, analog variations are being, and have been, synthesized and tested in animal tumor systems. Figure 1 exhibits the structures of some of the most active drugs found. In the course of these investigations, particularly with regard to structure-activity relations, a number of simple "rules of thumb" have surfaced. These are: (*a*) the complexes exchange only some of their ligands rapidly; (*b*) the complexes are neutral (although the in vivo biologically active form may be charged after ligand exchange has occurred); (*c*) the geometries of the complexes are either square planar or octahedral; (*d*) two *cis*-monodentate (or one bidentate) leaving groups are required (generally, the corresponding *trans* isomers of the monodentate leaving groups are inactive); (*e*) the rate of exchange of the leaving groups falls into a restricted "window of lability" centered roughly on that of the chlorides of the parent drug; (*f*) the leaving groups are spaced with atomic centers approximately 3.3 Å apart; and finally (*g*) the ligands *trans* to the leaving groups preferably

[1] Supported by grant CA-11349 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare; by research contracts and fellowship funds from Engelhard Industries and Matthey Bishop Inc.; and by a fellowship fund from the International Nickel Corp.

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[4] *Cis*-dichlorodiammineplatinum (II): CAS reg. No. 15663-27-1.

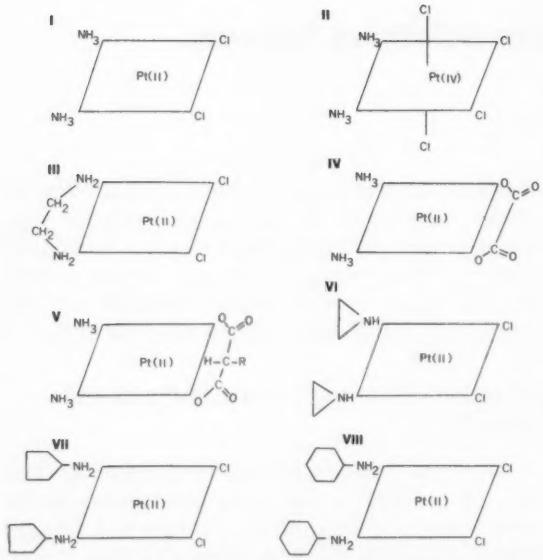

FIGURE 1.—Structural formulae of some representative active antitumor complexes of platinum: I = *cis*-dichlorodiammineplatinum(II); II = *cis*-tetrachlorodiammineplatinum(IV); III = dichloroethylenediamineplatinum(II); IV = oxalatodiammineplatinum(II); V = substituted (R) malonatodiammineplatinum(II); VI = *cis*-dichloro(bisethyleneimine)-platinum(II); VII = *cis*-dichloro(biscyclopentylamine)platinum(II); VIII = *cis*-dichloro(biscyclohexylamine)platinum(II).

should be strongly bonded relatively inert amine groups.

While initial chemical emphasis was placed upon variations on the nature of the leaving groups, recent evidence indicates that changes in the strongly bonded amine groups can affect extensive changes in the biologic activity. The fact that the corresponding *trans*-complexes to active *cis*-complexes are not active antitumor agents has provided a potent tool for investigating the biochemical interactions leading to antitumor activity. At this time, it would appear that platinum(II) complexes are being adequately investigated. Platinum(IV) complexes are still largely unexplored with regard to antitumor activity. This is also true for other platinum-group metals, and, indeed, there is almost a dearth of investigation into the activity of other metal coordination complexes. I would recommend that these latter groups require more attention at this time since we have no strong reason to believe that platinum complexes are unique. It was, therefore, a pleasure to participate in a symposium in which some attention was paid to the rhodium(II) complexes by Drs. Bear and Kimball et al (10,11), and to the group IIIa elements by Dr. Adamson et al (12).

Biologic Activity of Platinum (II) Complexes

The initial hint that platinum complexes may exhibit interesting biologic activity was the observation that at low concentrations some of these complexes were capable of completely inhibiting cell-division processes in a number of different bacteria without inhibiting growth, thus leading to filamentation. While many chemicals and physical treatments are known to cause bacterial filamentation, there are some unique features when this is caused by platinum complexes. For example, DNA synthesis in bacteria is not markedly inhibited at concentrations sufficient to cause filamentation, and filaments contain continuous strands of DNA (13) (*also* Drobník J, unpublished results). If the platinum complexes are removed from the growth medium, the filaments rapidly revert to normal colonies of *Escherichia coli*. Filamentation caused by platinum complexes is not a terminal event in *E. coli*. It is terminal when it is caused by bifunctional alkylating agents which do inhibit DNA synthesis. In tests of a variety of complexes for the correlation of antitumor activity and bacterial filamentation, it was at first thought that the two biologic actions were strongly correlated. However, Reslova and Drobník, and later others, have found exceptions to this rule, and the complete isomorphism between the two classes of biologic effects has broken down. A relatively new bacteriologic effect of the platinum complexes was discovered by Reslova (14); these complexes are able to induce lysis in lysogenic bacteria.

Up to the present, we have found no exception to the following general rule: complexes which are active antitumor agents are active inducing agents of lysogenic bacteria. These results provide a clue to the mechanism of action on a cellular level that may, with some caveats, be

extended to mammalian cells. Platinum complexes may be able to derepress totally the latent viral genomic information in cells, leading to the production of actively antigenic virions or virally coded proteins. Vonka et al have tested for these effects using Epstein-Barr viruses in human lymphocytes and they have found evidence consistent with the extension of the hypothesis to mammalian cells in tissue culture (15). More recently, Davidson, in our laboratory, has discovered a similar effect in an in vivo system (personal communication).

Tissue culture studies by Harder and Rosenberg (16) and simultaneous in vivo-in vitro studies by Howle and Gale (17) have determined that the primary action at the cellular level of treatment with concentrations of the platinum drugs equivalent to that found in therapeutically treated animals is a severe and prolonged inhibition of new DNA synthesis, with a minimal effect upon RNA and protein synthesis. Further, it was shown that the platinum treatment had little effect upon the synthesis or transport of precursors to the DNA. Therefore, the primary lesion in the cell caused by the platinum complex is an attack upon DNA. Subsequent studies by Harder and others (18–21) investigated the nature of these lesions. A multiplicity of possible platinum complex-DNA reactions has been uncovered. These include intrastrand and interstrand cross-linking and monodentate and bidentate attack upon various individual bases (22). This is an active area of research at the present time, and, as yet, we have not been able to identify any single type of lesion as leading to the antitumor effect. Nor, indeed, has it been shown whether any or all of these types of lesions are involved. The subject of metal and metal coordination complex interactions with DNA is an important new field of investigation for this and a number of other reasons.

The inhibition of DNA synthesis occurs at levels not frankly cytotoxic to the cells. During the inhibition phase, the cells often manifest giant cell formation, which progresses to a multinuclear stage and then to a final division into normal cells. Thus, it is possible that the primary lesions can be reversed or repaired by the cells. The studies of the involvement of repair mechanisms are still in a primitive stage.

We believe that the lability of the leaving groups is important in that the molecule should remain intact and nonreactive while being distributed through the host's body. The high chloride concentration of the blood, lymph, and interstitial fluids is sufficient to limit the hydrolysis of the chlorides on the complexes. Upon entering the cell by a passive diffusion process, the complex is exposed to a chloride ion concentration in the interior of the cell which is lower by a factor of 30 than the exterior, and hydrolysis of the chloride leaving groups can occur. This provides two active ligand attachment sites to the nuclear DNA. Bidentate leaving groups (such as malonate) are slower in their kinetic rate of hydrolysis by orders of magnitude, and these may require the intervention of cellular reactions to activate the molecules.

To establish the credentials of the platinum complexes as potent antitumor agents, I have compiled the best results of many different laboratories' tests using *cis*-dichlorodiammineplatinum(II) (table 1). The results show that this platinum complex is a broad-spectrum antitumor agent against transplantable, chemically induced, and virally induced tumors in different animal hosts. The potency can be estimated from the fact that in some tumor systems, the drug can be given to animals a few days prior to death from advanced tumors and still produce cures.

It is difficult to avoid the question as to why large tumors disappear so readily in so many systems at dose levels which are not frankly cytotoxic to tumor cells and which do not appear to be selectively accumulated in tumor tissue (23,24). Biochemical differences between tumor cells and normal cells are generally quantitative differences, and usually are not of a magnitude that suffices to ensure selectivity or specificity of drug action. Thus, cancer chemotherapy lacks an adequate concept of specificity, if one assumes that the actions of drugs are purely cytotoxic events. Specificity is inherent, however, in the immune reactions of host animals, and recommends itself as a dominant component in selective tumor destruction. At

TABLE 1.—Best results of the antitumor activity of *cis*-dichlorodiammineplatinum (II) in animal systems

Tumor	Host	Best results*
Sarcoma 180, solid	Swiss white mice	T/C = 2%
Sarcoma 180, solid (advanced)	Swiss white mice	100% cures
Sarcoma 180, ascites	Swiss white mice	ILS = 63%: 3/12 long-term survivors
L1210 leukemia	BDF_1 mice	ILS = 340%: 4/10 cures
Primary Lewis lung carcinoma	BDF_1 mice	100% inhibition
Ehrlich ascites	BALB/c mice	ILS = 300%
Walker 256 carcinosarcoma (advanced)	Fischer 344 rats	100% cures: TI >50
Dunning leukemia (advanced)	Fischer 344 rats	100% cures
P388 lymphocytic leukemia	BDF_1 mice	ILS = 293%: 4/6 cures
Reticulum cell sarcoma	C+ mice	ILS = 141%
B16 melanoma	BDF_1 mice	ILS = 240%: 8/10 cures
ADJ/PC6A myeloma	BALB/c mice	100% cures: TI = 8
AK leukemia (lymphoma)	AKR/LW mice	ILS = 225%: 3/10 cures
Ependymoblastoma	C57BL/6 mice	ILS = 141%: 1/6 cures
Rous sarcoma (advanced)	15-I chickens	65% cures
DMBA-induced mammary carcinoma	Sprague-Dawley rats	77% total regressions: 3/9 free of all tumors
ICI 42,464-induced myeloid and lymphatic leukemias	Alderly Park rats	ILS = 400%
MCDV-12 leukemia	BALB/c mice	ILS = 146%: 2/3 cures

*T/C = treated/control; ILS = increase in lifespan; TI = therapeutic index.

this time it is difficult to see how the various known classes of antitumor agents which are generally considered cytotoxic, can successfully stimulate a specific immune attack upon the tumor. This difficulty is compounded by the fact that most antitumor agents are also immunosuppressors. Our earlier clue that the antitumor-active platinum complexes are capable of derepressing virally coded information in bacteria and in a mammalian cell line suggested

to us that the antigenicity of the tumor may be altered by chemotherapy in favor of the host's ability to destroy the tumor. We have called this the "enhanced antigenicity" hypothesis (25). I should like to describe here some of the operational results supporting such a hypothesis. First, in our earlier screening of a variety of metal coordination complexes for antitumor activity against the solid Sarcoma 180 in Swiss Webster mice, we found a number of compounds that markedly and consistently produced an increase of the tumor size in the treated animals over the control animals (sometimes as much as 200%). This was interpreted as a drug-induced impairment of the host's immune competence, which normally was able to inhibit the rate of tumor growth, but without a concomitant effect upon the tumor. Second, there is a large body of information that animals cured of large tumors by chemotherapy retain a long-lasting immunity against this tumor and completely reject reimplants. Third, tumor cells treated with chemicals such as *cis*-dichlorodiammineplatinum(II) and platinum-pyrimidine complexes (*see* below) at concentrations two orders of magnitude lower than that which is frankly cytotoxic to the cells, retain a high viability count of the cells as measured by the fluorescein diacetate technique. These cells, when transplanted to a virgin host, do not produce tumors, and yet the hosts develop an immunity against further reimplants of nontreated tumor cells. Fourth, in a system where the drug normally can regress advanced solid Sarcoma 180 with close to 100% efficacy, Conran has found that hydrocortisone injections, which are known to depress the immune competence of the host, decreased the number of cures produced by the platinum drugs. Conversely, in a syngeneic system, such as Sarcoma 180 in the BALB/c mouse, where the platinum drug alone did not cause any regressions of the advanced tumors, animals which were injected earlier with zymosan, a known specific immune stimulant, showed a tumor cure rate of 40%–50% (26). Finally, Connors et al (27) and Tobe[5] have reported that the analog, *cis*-dichloro(biscyclopentylamine)platinum(II), has a therapeutic index over 500 against the ADJ/PC6A myeloma in mice. In the absence of any information concerning selective uptake of this drug by this tumor-host system, such specificity can only make sense in the context of an immune reaction.

Taken together, these results form the basis for the suggestion that the action at the animal level for the antitumor activity of the platinum complexes is the enhancement of the antigenicity of the tumor system which allows the immune system to mount a successful attack upon the tumor. Thus, the cellular studies have led to the reasonable conclusion that the primary lesion in the cell is a reaction of the platinum complex with DNA, while whole animal studies have led to the equally reasonable conclusion that the tumor disappears due to the successful intervention of the host's immune system. This raises the problem of how to bridge the hiatus between these two conclusions. In recent months, a new bridging hypothesis has emerged from our laboratory work and I should like to discuss this in some detail here. In order to do so we must return to some additional chemistry, that of the "platinum-pyrimidine blues."

"Platinum-Pyrimidine Blues"

During the course of an investigation of the interaction of *cis*-diaquodiammineplatinum(II) with various bases, nucleosides, nucleotides, and polynucleotides, Mansy et al[6] discovered a striking color change when polyuracil was mixed with the platinum aquo species; the solution turned deep blue within 24 hours at 37°C. The resulting product could not be crystallized from the solution, and the amorphous blue powder resulting from evaporation to dryness was highly soluble in water. We then found that uracil, thymine, other substituted pyrimidines, and pyrimidine nucleosides and nucleotides produce the same type of deep blue solution when reacted with *cis*-diaquodiammineplatinum(II) (28). The products have in common the characteristics of high solubility in water, complete amorphousness of the pre-

[5] Tobe ML. Presentation at the British Chemical Society Symposium, 1973.

[6] Mansy S, Rosenberg B, and Getty R. Raman spectra of solid platinum(II) polynucleotide complexes. Submitted for publication.

cipitated material, and extinction coefficients (calculated for a molecular weight of 400) on the order of 300–1000 for the absorption bands in the wavelength region of 570–750 nm. These blue products were tested for antitumor activity since they contained the active moiety of the diammineplatinum structure. They proved to be a subclass of highly potent antitumor agents against a broad spectrum of different tumor-host systems. An additional benefit derived from these complexes when compared to *cis*-dichlorodiammineplatinum(II) was the lack of kidney toxicity as the dose-limiting side effect. For the following reasons these "platinum blues" are an interesting subclass for further investigation: (*a*) high solubility, (*b*) low toxicity, (*c*) minimal kidney toxicity, and (*d*) high antitumor activity. A comparison of the activity of a number of different "platinum blue" complexes with that of *cis*-dichlorodiammineplatinum(II) against ascites Sarcoma 180 in Swiss Webster mice is shown in table 2. Further studies in the preclinical stages are presently under way.

The "platinum-pyrimidine blues," as well as the original "Platinblau" described by Hofmann and Bugge (29), present a difficult problem with regard to structural analysis because of the inability to crystallize any of these materials. In addition, chemical analysis of many such complexes can only be rationalized if the material consists either of mixtures or of nonstoichiometric complexes. We cannot at this stage answer any of the following questions: (*a*) Are these complexes monomeric or oligomeric in solution? (*b*) Is the platinum still in the II valence state or is it oxidized to the IV valence state, or a mixture of the two? (*c*) Are these species in solution charged or neutral, ie, are they salts? (*d*) Is the appearance of the absorption band in the red region with its low extinction coefficient due to a perturbed d-d electronic transition of the platinum atom or is it due to ligand charge transfer? If it is the former, is the electronic energy perturbation due to a platinum-platinum interaction or a platinum-ligand interaction? (*e*) Does the platinum bind to the pyrimidine moiety via two bonds causing a closed ring or via one bond with some other ligand in the fourth position?

TABLE 2.—Survey results of antitumor activity of platinum blue complexes against ascites Sarcoma 180 in Swiss mice

Compound	Dose range (mg/kg)	Toxic level (mg/kg)	Best %ILS	Dose of best %ILS (mg/kg)	No. of cures/ No. of animals treated
Untreated control*					
Positive control [*cis*-dichlorodiammine-platinum (II)]	7	10	49 (SD ± 2.82)	7	1/12
Platinum complexes					
Uracil	50–400	400	91	200	5/6
5,6-Dihydrouracil	20–800	400	92	200	4/6
Thymine	150–600	450	72	300	2/6
5,6-Dihydro-6-methyluracil	50–400	200	89	50	2/6
6-Methyluracil	200–800	>800	87	600	3/6
5,6-Dimethyluracil	50–400	>400	100	400	5/6
5,6-Dihydrothymine	50–400	400	87	200	2/6
1-Methyluracil	50–400	>400	85	400	3/6
1-Methylthymine	50–400	400	94	100	3/6
1-Ethyluracil	50–400	200	38	50	0/6
5-Fluorouracil	50–400	200	90	100	4/6
5-Chlorouracil	50–400	200	67	50	3/6
6-Chlorouracil	25–200	>200	88	200	3/6
5-Bromo-1-methyluracil	50–400	>400	88	200	1/6

*Average day of death = 17.5 (SD ± 2.16).

These basic questions, and numerous others, cannot yet be resolved with any certainty.

In our laboratory, Pant investigated the in vitro interaction of the "platinum blues" with various nucleic acids. It was immediately evident that they react with great avidity, causing a precipitate of deep blue-stained fibers of DNA or RNA. The reactions are very rapid and the kinetics are presently being studied. The "platinum blues" interact very poorly with proteins. G. Petsko (personal communication) found no evidence for an interaction of the platinum-uracil blues with crystalline ribonuclease S or lysozyme. *Cis*-dichlorodiammineplatinum(II) does react readily with specific amino acids of both of these proteins. On the basis of Petsko's evidence, the kinetics of the interaction of the "platinum blues" with these proteins appear to be slower by two to four orders of magnitude.

Taking a clue from this nucleic acid selectivity in vitro of the "platinum blues," Wagner in our laboratory attempted to stain cells with these complexes to study the distribution using light microscopy. The cells stained, but the blue color was only marginally detectable. Electron-microprobe studies, however, did indicate that the platinum was primarily localized in the cell nucleus. Since platinum is a suitable heavy atom for electron microscopy, Aggarwal et al (30) investigated the stained cells in a transmission electron microscope. The "platinum blue" was the only heavy atom used. It was immediately successful. The cell pictures showed high electron density in the chromatin and nucleolus in the nucleus, and in the ribosomes in the cytoplasm, all nucleic acid sites in the cell. No membrane structures were visible except as negatives outlined by nucleic acid-containing structures. Proteins and lipids were not stained. These studies show that the "platinum blues" are selective (or even specific) stains for nucleic acids in cells (30), as predicted by Pant's in vitro results.

Cell Surface-Associated Nucleic Acids in Tumorigenic Cells

We then proceeded to stain a number of different cell types with the platinum-thymine blue complex. These included tumor cells (eg, Sarcoma 180 [both solid and ascites], L1210, Ehrlich ascites, MOPC-21 myeloma, rat embryonic skin [RESI], etc), continuous cultures (eg, HeLa, AV_3, human lymphoblastoid, KB, V-79, etc), and normal cells, both primary lines in culture and extracted tissue. It quickly became obvious that an additional staining site occurred in tumorigenic cells, but not in normal or weakly or nontumorigenic transformed cells. This was the appearance of very densely stained patches associated with the cell plasma membrane. One such cell is shown in figure 2. Of the 19 cell types investigated, the correlation of the appearance of these surface patches with tumorigenicity of the cell was surprisingly complete. (There is, of course, some ambiguity in determining the tumorigenicity of transformed cell lines such as HeLa and AV_3. But it is clear that not all transformed lines are capable of growing into tumors in an immunocompetent host, and at present there are no completely acceptable biologic markers which segregate tumorigenic from nontumorigenic transformed cell lines.)

We attempted to characterize the nature of the cell surface-associated stained sites by specific enzyme predigestion (for times and at concentrations which yielded 50%–80% viable cells) to determine whether specific enzymes prevented the appearance of the stained patches after subsequent fixation and staining. For the ascites Sarcoma 180 cells, only neuraminidase and DNase I eliminated the appearance of the stained patches; trypsin, hyaluronidase, RNase, and concanavalin A did not. Since neuraminic (sialic) acid occurs on normal cell membranes, which do not show such surface staining, it cannot be the site of reaction with the platinum-thymine blue. The success with DNase I identifies the stained patches on these cells as DNA. It is a likely interpretation of these results that this DNA is bound to the cell surface through association with neuraminic acid. From this preliminary work, we tentatively conclude that tumorigenic cells have DNA associated with the cell surface, while nontumorigenic cells do not (with the exception of thymocytes which show surface staining of a different order) (31). This raises some interest-

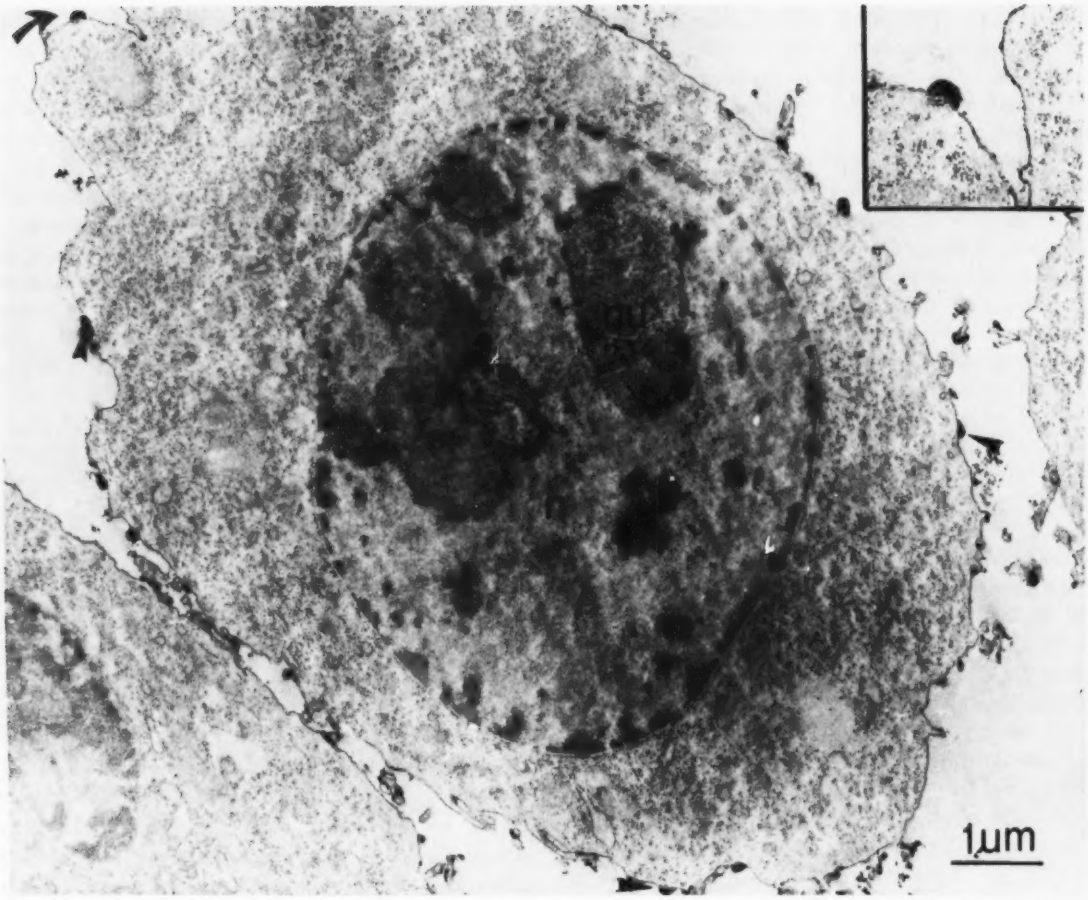

FIGURE 2.—Electron micrograph of an ascites Sarcoma 180 cell treated with the platinum-thymine complex showing staining only in the nucleolus (nu) and chromatin material in the nucleus (n), and in the ribosomes in the cytoplasm. Note the presence of deeply stained surface patches at the plasma membrane (arrows). One such patch (curved arrow) is enlarged (4 ×) in the inset for clarity. [Reproduced from *Proc Natl Acad Sci USA* (*see* ref 31)].

ing questions, the answers to which are being intensively pursued.

Speculation on the Role of Surface-Associated Nucleic Acids

Even without any understanding of the role of the surface-associated nucleic acids, our results suggest the following possible conclusion: cell transformation is a necessary but not sufficient condition for tumorigenicity; the appearance of nucleic acid at the cell surface provides for sufficiency. This is a testable conclusion, and a number of experimental tests of its validity are under way.

Nucleic acids have two particular characteristics of interest here: they are the most basic information-carrying molecules and they are the weakest antigenic biopolymers. If their role on the surface depends on the former characteristic, we could call it a "functional" mechanism; if the latter, it would be a "trivial" mechanism. The functional mechanism may involve viral

origin of the nucleic acid and possibly cell-to-cell infectivity. As yet, we have no information concerning these and I will not speculate further here. With regard to the trivial mechanism, there are some comments possible. Since transformed cells can, with a great deal of success, be grown in immunologically privileged sites in animals, such as the hamster cheek pouch or the anterior chamber of the eye, or in immunologically deprived animals (thymectomized, irradiated, or nude mice), but cannot always be grown in immunologically competent animals, it is apparent that not all transformed cells can escape immunologic surveillance in animals. Transformed cells produce new antigens at the cell surface, which generate a host immune reaction leading to cell destruction. If, however, some transformed cell types can mask the strong antigens with the relatively much weaker antigens such as nucleic acids, they can escape the host immune surveillance and form a tumor clone. A relatively simple extension of this idea then provides us with the desired bridging hypothesis mentioned earlier. The primary lesions on the DNA caused by the platinum complexes (either in the nucleus or at the surface) lead to the disruption of the antigen masking, and consequently to the exposure of the strong antigens on the cell surface (the "enhanced antigenicity").

Attractive as this idea is, I would not have ventured so deeply into speculation if it did not have a potentially significant corollary for chemotherapy: if the drugs can react with the surface nucleic acids to disrupt their masking function, then they need not enter the cell at all. This raises the possibility of a new class of drugs designed (*a*) to be excluded from penetrating cell membranes but reactive with nucleic acids, (*b*) to be specific in their actions on tumor cells (and perhaps thymocytes as well) since these are, so far, the only cells shown to have nucleic acids on their surfaces, but (*c*) not to be cytotoxic to normal cells.

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Studies on the Antitumor Activity of Gallium Nitrate (NSC-15200) and Other Group IIIa Metal Salts [1]

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SUMMARY

Several group IIIa metal salts, eg, aluminum nitrate, gallium nitrate, indium nitrate, and thallium chloride, have been evaluated for in vivo toxicity in mice and rats, for cytotoxicity in tumor cells in vitro, and for activity against a broad spectrum of experimental rodent tumors. The position of these agents in the periodical table roughly parallels their toxicity, the LD50s decreasing with increasing atomic weights. This parallel also exists with regard to in vitro cytotoxicity to Walker 256 carcinosarcoma cells. Although all of the metal salts had activity against the ascites Walker 256 carcinosarcoma, they were ineffective in ascites leukemias, plasma cell tumors, or Ehrlich carcinoma. Gallium nitrate was particularly active against solid tumors transplanted subcutaneously, suppressing the growth of six of eight tumors more than 90%. Because of its demonstrated antitumor activity in rodents and its uptake and concentration by various animal and human tumors, gallium nitrate has potential usefulness in the treatment of solid tumors in man and has been entered into a phase I study at the National Cancer Institute.

[Cancer Chemother Rep 59:599–610, 1975]

Since the reports by Rosenberg et al (1,2) that various complexes of group VIII metals such as platinum possess antitumor activity against some experimental animal tumors, interest in the potential usefulness of metal complexes and salts in cancer chemotherapy has been increasing. Indications that the group IIIa metal salts might have promise as antitumor agents were derived from several observations including those of Edwards and Hayes (3), who reported that carrier-free gallium^{67}-citrate was localized in non-osseous tumors such as Hodgkin's disease and other lymphomas. It was subsequently found that this compound is deposited in other tumors as well, including poorly differentiated thyroid adenocarcinoma, reticulum cell sarcoma, lymphosarcoma, and carcinomas of the lung, breast, colon, and kidney (3–7). Parallel studies in animals showed that gallium^{67} was localized in seven non-osseous rodent carcinomas and sarcomas, but that it was not deposited in normal tissue (8). In addition, Drug Research and Development (formerly the Cancer Chemotherapy National Service Center), DCT, NCI, found that the nonradioactive nitrate salt of gallium

[1] Gallium nitrate: CAS reg. No. 13494-90-1.

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had antitumor activity against Walker 256 carcinosarcoma in rats.

These findings prompted us to examine the toxicity of some of the group IIIa metal salts in rodents, to assess the in vitro cytotoxicity of these compounds, and to evaluate them with regard to their in vivo antitumor activity against a broad spectrum of experimental tumors in rodents. Some of these studies have been reported previously (9,10).

MATERIALS AND METHODS

Drugs

The group IIIa metal salts were obtained from the Alfa Chemical Company, Beverly, Massachusetts, and were used without further purification. The drugs were dissolved in 0.9% NaCl and administered by intraperitoneal (ip) or subcutaneous (sc) injection in a volume of 0.01 ml/g of body weight.

In Vitro Cytotoxicity Studies

L1210 leukemia and Walker 256 carcinosarcoma cells were maintained in static culture in RPMI 1630 media. The media were supplemented with 20% (L1210) or 10% (Walker 256) fetal calf serum. Stock cultures were counted and diluted so that there were approximately 10^5 cells/ml with a total of 5 ml in each bottle. Drugs were dissolved in saline and filtered through a 0.45μ Millipore filter immediately before use. A total of 0.1 ml of the drug solution or saline alone was added to 5 ml of the cells. The pH of the saline to be added to each control bottle was adjusted with 0.1N HCl so that it was identical to that of the drug solutions. Cell counts were made every 24 hours for 3 days with a Coulter counter. The effect of the metal salts on the growth of each cell line was determined at least twice with duplicates at each concentration, and the results are presented as the ID50 (the concentration of drug necessary for 50% inhibition of growth 72 hours after exposure), calculated according to the method of Litchfield and Wilcoxon (11).

In Vivo Toxicity and Antitumor Activity Studies

Adult (20–25 g) CDF_1 mice and adult (120–150 g) Sprague-Dawley rats were randomly divided into groups of eight to ten. The 30-day lethality of aluminum nitrate, gallium nitrate, indium nitrate, and thallium chloride was determined after the ip injection of daily doses of each salt for 10 consecutive days to groups of ten mice or eight rats.[5] Body weights of all animals were recorded daily, and the mean and standard error of the mean (SEM) were calculated for each group. The LD50 with its 95% confidence interval, the slope of the log dose, the percent mortality curve, and the LD10 were calculated by the method of Litchfield and Wilcoxon (11). Animals showing signs of marked toxicity between Days 4 and 30 were killed, autopsied, and examined for gross and microscopic evidence of toxicity.

For antitumor activity studies, tumor-bearing mice and rats were randomly divided into groups of ten and six respectively. Tumors were transplanted ip by dilution of ascites fluid from donor animals or were transplanted sc by trocar using fragments of solid tumors from donor animals. Table 1 summarizes the tumor cell types inoculated, the species and strain of the host, the route of tumor transplantation, and the dilutions used for the ip transplanted tumors. Beginning 24 hours after inoculation, each animal was given a daily ip injection of one of the test compounds for 10 consecutive days. The dose of each compound tested was $\leq$ its LD10 in that species. Control animals received daily injections of saline, the pH of which had been adjusted with 0.1N HCl to that of the drug solution. Each dose of each drug was administered in at least two separate experiments for each tumor. The average ± SEM of body weights of each group of animals was recorded daily during the first 10 days following tumor transplantation and weekly thereafter. The activity of the salts against the ip transplanted tumors was estimated by calculating the percent increase in the median survival time (MST) of drug-treated animals as compared to the saline-treated controls; the activity of the salts against the tumors transplanted

[5] Aluminum nitrate: NSC-143017; CAS reg. No. 10487-59-9; nitric acid, aluminum salt, nonahydrate.
Indium nitrate: NSC-15202.
Thallium chloride: NSC-15197; CAS reg. No. 7791-12-0.

TABLE 1.—Summary of tumor transplantations in rodents

Tumor cell type	Host		Route of transplantation	Dilution*
	Species	Strain		
Walker 256 carcinosarcoma	Rat	SD	Ip	1:100
Walker 256 carcinosarcoma	Rat	SD	Sc	—
Leukemia L1210	Mouse	CDF_1	Ip	1:1000
Leukemia K1964	Mouse	CDF_1	Ip	1:10
Leukemia K1964	Mouse	CDF_1	Sc	—
Leukemia P388	Mouse	CDF_1	Ip	1:20
Plasma cell YPC-1	Mouse	(BALB/c × A/Ln)f_1	Ip	1:1000
Ehrlich ascites carcinoma	Mouse	NIH Swiss	Ip	1:10
Fibrosarcoma M-89	Mouse	C57BL/Ka	Sc	—
Adenocarcinoma 755	Mouse	C57BL/6N	Sc	—
Mammary carcinoma YMC	Mouse	CDF_1	Sc	—
Reticulum cell sarcoma A-RCS	Mouse	CDF_1	Sc	—
Osteosarcoma 124F	Rat	Fischer	Sc	—
Lymphosarcoma P1798	Mouse	BALB/c	Sc	—

*Ip transplantation of tumors was performed by diluting the ascites fluid from a donor animal with Locke's solution; recipients received 0.1 ml of the resulting cell suspension. Sc transplantation of tumors was performed by trocar, using fragments of solid tumors from donor animals bearing the appropriate tumor. Tumors were transplanted from donors of the corresponding species and strain which had been inoculated with tumor cells 7–14 days previously.

sc was estimated by measuring the three dimensions of the tumors with calipers 10 days (for the Walker 256 carcinosarcoma) or 20 days (for the remainder of the tumors) after the initiation of daily drug treatment. Autopsies were performed on all rodents surviving 4 months after tumor inoculation, and those with no evidence of tumor are referred to as long-term survivors.

DNA Polymerase Assays

Reverse transcriptase from Rauscher murine leukemia virus was a gift of Dr. John Abrell and was purified as described previously (12). The DNA polymerase from Walker 256 carcinosarcoma was prepared and purified as reported by Waalkes et al (13). The standard assay mixture in 0.05 ml contained 50 mM Tris-HCl (pH 7.6); 5 mM dithiothreitol; 0.8 mM deoxynucleotide triphosphate (dATP, dGTP, and dCTP); 50 mM NaCl; and either 5 mM $MgCl_2$ or 1 mM $MnCl_2$. In addition, template-primers were used at 35–50 μg/ml and the concentration of TTP-^{3}H was 10^{-6} M (1000–1500 cpm/pmol). Incubations were carried out at 37°C. Acid-precipitable radioactivity was collected and washed on Whatman No. 1 filters by the technique of Bollum (14). Protein determinations were made by the method of Lowry et al (15). The enzyme (15 μl) was preincubated with gallium nitrate for 15 minutes at room temperature, and then template and substrates were added. The mixture was incubated for 30 minutes, during which time the kinetics of polymerization were linear. Preferred templates were used in all experiments. These templates were: activated DNA for polymerase A, $dT_{12-18} \cdot rA$ for polymerase B and that from the

Rauscher leukemia virus, and $dT_{12-18} \cdot dA$ for polymerases C and D.

RESULTS

Table 2 summarizes the toxicity of group IIIa metal salts in mice, and shows these salts arranged in order of increasing atomic weights. Their toxicity, as measured by the LD50 values, increased in parallel with their atomic weights, although the difference between the LD50 values for the two heaviest salts was slight. The lethality observed with all of the salts was accompanied by a variable degree of weight loss, an effect which did not appear to be dose related. Qualitatively similar results were obtained in toxicity studies in the Sprague-Dawley rat (table 3), although the LD50 of thallium chloride was slightly greater than that of indium nitrate. With the exception of aluminum nitrate, increasing doses of the metal salts resulted in an increasingly severe depression of body weight. This apparent lack of effect of aluminum nitrate on body weight, however, may have been due to the chemical peritonitis with consequent ascites formation which was the most striking manifestation of its toxicity in rats.

The major pathologic findings following treatment with the group IIIa metal salts are summarized for mice and rats in table 4. In mice, pulmonary damage was a common finding for all four of the metal salts tested. Following treatment with gallium nitrate, renal tubular damage was also noted, and mice injected with indium nitrate showed spermatogenic arrest and bone marrow hyperplasia. All of the metal salts caused pneumonitis in rats, and rats treated with gallium nitrate showed liver and kidney damage; indium nitrate induced liver but not kidney lesions. Alopecia developed in both mice and rats treated with thallium chloride about 20 days after initiation of treatment, an effect not observed with the other metal salts tested.

TABLE 2.—Toxicity of group IIIa metal salts in mice

Compound	LD50* (mg/kg)	LD10 (mg/kg)	Slope†	Dose (mg/kg)	% Lethality‡	Weight change§ (g)
Aluminum nitrate	320 (282–374)	213	1.40	0	0	+2.1
				250	30	−5.4
				300	40	−4.9
				350	60	−4.6
				400	70	−6.0
Gallium nitrate	80 (67.2–95.2)	63	1.22	0	0	+2.1
				75	40	−6.9
				80	50	−6.0
				90	70	−6.1
Indium nitrate	7.95 (5.87–9.40)	4.22	1.62	0	0	+2.1
				4	10	−2.4
				5	20	−5.9
				7.5	75	−8.0
Thallium chloride	6.9 (6.52–7.3)	6.0	1.13	0	0	+2.1
				6	10	+2.2
				6.5	30	−2.4
				7.5	70	−4.6
				8	80	−3.6

*Nos. in parentheses = 95% confidence interval.

†Slope of the log dose-percent mortality curve.

‡Percentage of all animals treated that died within 30 days of the first injection.

§Average weight change between Days 1 and 10 of drug administration. Mice were given daily ip injections for 10 days. The observation period totaled 30 days from the first injection.

TABLE 3.—Toxicity of group IIIa metal salts in rats

Compound	LD50* (mg/kg)	LD10 (mg/kg)	Slope†	Dose (mg/kg)	% Lethality‡	Weight change§ (g)
Aluminum nitrate	327 (283–378)	240	1.30	0	0	+20.2
				200	0	+18.5
				300	37.5	+22.0
				350	62.5	+19.5
				400	87.5	+23.2
Gallium nitrate	67.5 (59.5–76.6)	50	1.19	0	0	+20.2
				50	12.5	−9.8
				62.5	37.5	−25.6
				75	62.5	−32.8
				87.5	87.5	−35.0
Indium nitrate	5.55 (4.66–6.60)	3.85	1.36	0	0	+20.2
				4	12.5	−20.8
				5	37.5	−27.2
				6	62.5	−37.6
Thallium chloride	5.66 (5.25–6.10)	4.85	1.13	0	0	+20.2
				5	14.3	+9.6
				6	71.5	−1.7
				6.5	87.8	−19.2

*Nos. in parentheses = 95% confidence interval.
†Slope of the log dose-percent mortality curve.
‡Percentage of all animals treated that died within 30 days of the first injection.
§Average weight change between Days 1 and 10 of drug administration. Rats were given daily ip injections for 10 days. The observation period totaled 30 days from the first injection.

TABLE 4.—Major pathologic findings following treatment with group IIIa metal salts*

Compound	Pathologic findings in the —	
	Mouse	Rat
Aluminum nitrate	Congestion of liver and lungs	Congestion of lungs; chemical peritonitis with ascites formation
Gallium nitrate	Pneumonitis and chronic inflammatory infiltration of alveolar septa and capillaries; renal tubular damage	Chemical peritonitis; interstitial pneumonitis with pleural effusion; liver and kidney damage
Indium nitrate	Hepatic myelopoiesis; interstitial pneumonitis; spermatogenic arrest; bone marrow hyperplasia; congestion or hemorrhage in peritoneum and/or pleural cavity	Interstitial pneumonitis and inflammation of hepatic serosa and parenchyma
Thallium chloride	Pneumonitis; alopecia	Pneumonitis; alopecia

*Groups of 10 mice or 8 rats were given daily ip injections of varying doses of each salt for 10 consecutive days. Animals showing signs of marked toxicity between Days 4 and 30 were killed, autopsied, and examined for gross and microscopic evidence of toxicity.

Table 5 compares the in vitro cytotoxicity of the group IIIa metal salts in Walker 256 carcinosarcoma and L1210 leukemia cells. The former cells appear to be much more sensitive to all four of the salts tested than the L1210 leukemia cells, the ID50 decreasing in parallel with increasing atomic weights. In L1210 leukemia cells, the ID50 values for aluminum nitrate, gallium nitrate, and indium nitrate are approximately 4, 2, and 20 times greater, respectively, than in Walker 256 cells; the ID50 values for thallium chloride are similar in the two cell types.

Table 6 shows the in vivo antitumor activity of aluminum nitrate against both ascitic and solid transplantable tumors of rodents. Aluminum nitrate was an effective inhibitor of the ip transplanted Walker 256 carcinosarcoma, with only minimal activity against P388 leukemia and no activity against L1210 leukemia, K1964 leukemia, plasma cell YPC-1, or Ehrlich ascites carcinoma. Aluminum nitrate increased the MST of rats inoculated with the Walker 256 carcinosarcoma by the ip route 300% as compared to saline-injected controls, and 42% of the treated tumor-bearing rats were long-term survivors. When administered to rats carrying the sc transplanted Walker 256 carcinosarcoma, however, aluminum nitrate depressed tumor growth only 6% as compared to saline-injected controls. Although aluminum nitrate was more effective against various other sc transplanted tumors (eg, fibrosarcoma M-89, Adenocarcinoma 755, mammary carcinoma YMC, reticulum cell sarcoma A-RCS, lymphosarcoma P1798, and osteosarcoma 124F), it never depressed tumor growth more than 68% of controls.

TABLE 5.—In vitro cytotoxic activity of group IIIa metal salts against Walker 256 carcinosarcoma and L1210 leukemia cells

Compound	ID50* in—	
	Walker 256 carcinosarcoma	L1210 leukemia
Aluminum nitrate	70 (4.90)	250 (16.5)
Gallium nitrate	58 (3.85)	100 (7.50)
Indium nitrate	35 (2.70)	700 (3.50)
Thallium chloride	6 (3.70)	8.2 (5.80)

*Concentration (normality $\times 10^4$) of drug necessary for 50% growth inhibition 72 hours after addition to culture as determined by the method of Litchfield and Wilcoxon (11). Nos. in parentheses = slope of the dose-response curve ($\triangle$% growth inhibition/$\triangle$ normality).

Although gallium nitrate was somewhat less effective than aluminum nitrate against the ascitic form of Walker 256 carcinosarcoma (table 7), it showed relatively high activity against the sc transplanted Walker 256 tumor and was active in most of the other solid tumors against which it was tested, inhibiting the growth of fibrosarcoma M-89, Adenocarcinoma 755, reticulum cell sarcoma A-RCS, lymphosarcoma P1798, and osteosarcoma 124F more than 90% of the controls.

Indium nitrate (table 8) showed only moderate activity against both ascitic and solid forms of the Walker 256 carcinosarcoma. Although the MST of rats carrying the ascitic form of this tumor was increased 124%, there were no long-term survivors; indium nitrate inhibited the growth of the solid form of the tumor to 39% of controls. Similar results were obtained when indium nitrate was tested for antitumor activity against other experimental solid tumors, the inhibition of tumor growth varying from 26% for the fibrosarcoma M-89 and osteosarcoma 124F to 55% for the reticulum cell sarcoma A-RCS.

Thallium chloride (table 9) showed the highest activity of any of the metal salts tested against the ascitic form of the Walker 256 carcinosarcoma, increasing the MST to greater than 700% that of controls; 25% of the tumor-bearing rats treated with thallium chloride were long-term survivors. In contrast, thallium chloride had no activity against the solid form of Walker 256, and showed no activity or only minimal activity in the remaining solid tumors against which it was tested.

Ancillary studies were performed to assess the antitumor activity of the group IIIa metal salts given by the sc as compared to the ip route, using rats carrying the ascitic form of the Walker 256 carcinosarcoma. Such experiments demonstrated that treatment of the ascitic tumors with the metal salts given by the sc route reduced their antitumor effectiveness. Thus, no increase in the MST was noted in rats

TABLE 6.—Effect of aluminum nitrate on the growth of various experimental animal tumors*

Tumors	Optimal antitumor dose (mg/kg)	Increase in MST (% of controls)	No. of long-term survivors/ No. of animals treated†	Growth inhibition‡ (% of controls)
Ip transplantation				
Walker 256 carcinosarcoma	150	300	5/12 (42)	
Leukemia L1210	100	0	0/12 (0)	
Leukemia K1964	100	0	0/12 (0)	
Leukemia P388	100	11	0/12 (0)	
Plasma cell YPC-1	100	0	0/12 (0)	
Ehrlich ascites carcinoma	100	0	0/12 (0)	
Sc transplantation				
Fibrosarcoma M-89	100			59
Leukemia K1964	100			0
Adenocarcinoma 755	150			42
Mammary carcinoma YMC	100			30
Reticulum cell sarcoma A-RCS	100			37
Lymphosarcoma P1798	100			48
Walker 256 carcinosarcoma	100			6
Osteosarcoma 124F	150			68

*Animals were injected ip daily for 10 days, beginning 1 day after tumor transplant.
†Nos. in parentheses = percent of long-term survivors.
‡Three-dimensional measurements of sc transplanted tumors were taken 20 days after transplant with the exception of the Walker 256 carcinosarcoma which was measured 10 days after transplantation.

treated with aluminum nitrate or thallium chloride by the sc route, whereas when given by ip injection these compounds increased the survival time 300% and 769% respectively (*see* tables 6 and 9). Gallium nitrate administered by the ip route increased the MST 138% over the saline-injected controls, whereas when the salt was given sc this value was reduced to 50%. Similarly, the MST was increased 124% over controls when animals were treated with ip injections of indium nitrate, but an increase of only 19% was noted following treatment by the sc route.

Table 10 shows the effect of anion substitution on the antitumor activity of group IIIa metal salts when tested against the ascitic form of the Walker 256 carcinosarcoma. The nitrate, chloride, and sulfate salts of aluminum all were effective in increasing the MST of tumor-bearing rats, with an increase of 550% noted following treatment with aluminum chloride; the greatest percentage (42%) of long-term survivors was found after treatment with the nitrate salt. Little difference was found among the nitrate, chloride, and sulfate salts of gallium with regard to percent increase in the MST, although as with aluminum, treatment with the nitrate salt resulted in a greater number of long-term survivors. Comparison of the nitrate, chloride, sulfate, and acetate salts of indium revealed that the nitrate salt effected the largest percent increase in MST and that none of the salts increased the number of long-term survivors. The acetate salt of thallium

TABLE 7.—Effect of gallium nitrate on the growth of various experimental animal tumors*

Tumors	Optimal antitumor dose (mg/kg)	Increase in MST (% of controls)	No. of long-term survivors/ No. of animals treated†	Growth inhibition‡ (% of controls)
Ip transplantation				
Walker 256 carcinosarcoma	60	138	4/12 (33)	
Leukemia L1210	50–60	0	0/12 (0)	
Leukemia K1964	50–60	0	0/12 (0)	
Leukemia P388	50–60	0	0/12 (0)	
Plasma cell YPC-1	50–60	0	0/12 (0)	
Ehrlich ascites	50–60	3	0/12 (0)	
Sc transplantation				
Fibrosarcoma M-89	50			95
Leukemia K1964	50			54
Adenocarcinoma 755	60			93
Mammary carcinoma YMC	50			24
Reticulum cell sarcoma A-RCS	50			94
Lymphosarcoma P1798	30			98
Walker 256 carcinosarcoma	60			92
Osteosarcoma 124F	40			96

*Animals were injected ip daily for 10 days, beginning 1 day after tumor transplant.
†Nos. in parentheses = percent of long-term survivors.
‡Three-dimensional measurements of sc transplanted tumors were taken 20 days after transplant with the exception of the Walker 256 carcinosarcoma which was measured 10 days after transplantation.

was less effective than thallium chloride both in increasing the MST of tumor-bearing rats and in producing long-term survivors.

Gallium nitrate, at a concentration of 50 μg/ml, inhibits DNA polymerases B and D from Walker 256 cells, as well as the DNA polymerase (reverse transcriptase) from Rauscher murine leukemia virus (table 11). The degree to which gallium nitrate inhibits the latter enzyme and the polymerases B and D from the Walker 256 cells is comparable to that seen with rifamycin SV:*O-n*-octyloxime, and greater than that observed following incubation with rifamycin SV.

DISCUSSION

Several of the group IIIa metal salts have been tested for toxicity in rodents, for cytotoxicity in vitro, and for in vivo activity against a number of experimental rodent tumor systems. In general, the toxicity of the metal salts in rats and mice increased in parallel with increasing atomic weights; this toxicity was manifested by a depression in body weight gain as well as pathologic changes in the lungs of all animals and in the liver and kidneys of some rodents. The compounds possessed greater in vitro cytotoxicity to Walker 256 carcinosarcoma than to L1210 leukemia cells; the reason for this differential cytotoxicity cannot be determined from our studies, but may be due to lack of uptake or concentration of the metal salts in the latter cells. However, it has been demonstrated that granulocytes from patients

TABLE 8.—Effect of indium nitrate on the growth of various experimental animal tumors*

Tumors	Optimal antitumor dose (mg/kg)	Increase in MST (% of controls)	No. of long-term survivors/ No. of animals treated†	Growth inhibition‡ (% of controls)
Ip transplantation				
Walker 256 carcinosarcoma	1	124	0/18 (0)	
Leukemia L1210	2–3	6	0/12 (0)	
Leukemia K1964	2–3	0	0/12 (0)	
Leukemia P388	2–3	0	0/12 (0)	
Plasma cell YPC-1	2–3	0	0/12 (0)	
Ehrlich ascites	2–3	0	0/12 (0)	
Sc transplantation				
Fibrosarcoma M-89	2			26
Leukemia K1964	3			27
Adenocarcinoma 755	3			33
Mammary carcinoma YMC	3			37
Reticulum cell sarcoma A-RCS	3			55
Lymphosarcoma P1798	2			30
Walker 256 carcinosarcoma	2			39
Osteosarcoma 124F	1			26

*Animals were injected ip daily for 10 days, beginning 1 day after tumor transplant.
†Nos. in parentheses = percent of long-term survivors.
‡Three-dimensional measurements of sc transplanted tumors were taken 20 days after transplant with the exception of the Walker 256 carcinosarcoma which was measured 10 days after transplantation.

with chronic granulocytic leukemia have a high affinity for gallium[67] (16).

The metal salts also had greater in vivo antitumor activity in the ip transplanted Walker 256 carcinosarcoma than in rapidly dividing leukemia cells such as L1210, K1964, and P388 leukemias; they were inactive in the plasma cell YPC-1 and Ehrlich ascites carcinoma. It is possible that they are ineffective, at least against L1210 leukemia, because the concentration required for in vitro growth inhibition of this cell line is toxic in vivo.

The group IIIa metal salts, gallium nitrate in particular, possessed greater activity against solid tumors transplanted sc than against ip transplanted ascitic tumors. Gallium nitrate was more than 90% effective against the solid form of Walker 256 carcinosarcoma, lymphosarcoma P1798, and Adenocarcinoma 755, its activity possibly deriving from its ability to concentrate in these tumors (8,17). Indium has also been reported to concentrate in these tumors, but to a lesser extent (17). When the salts were injected at a site differing from that occupied by the tumor, only gallium nitrate and indium nitrate were effective as antitumor agents, possibly because they are sequestered by the tumor from the general circulation.

The gamma-emitting isotope of gallium (Ga^{67}) has been utilized in man for whole-body scans, and has demonstrated usefulness in localizing abscesses (18) and tumors such as melanomas and lymphomas (8,19). Because of the demonstrated antitumor activity of gal-

TABLE 9.—Effect of thallium chloride on the growth of various experimental animal tumors*

Tumors	Optimal antitumor dose (mg/kg)	Increase in MST (% of controls)	No. of long-term survivors/ No. of animals treated†	Growth inhibition‡ (% of controls)
Ip transplantation				
Walker 256 carcinosarcoma	4	769	3/12 (25)	
Leukemia L1210	3	0	0/12 (0)	
Leukemia K1964	3	0	0/12 (0)	
Leukemia P388	3	0	0/12 (0)	
Plasma cell YPC-1	3	0	0/12 (0)	
Ehrlich ascites	3	0	0/12 (0)	
Sc transplantation				
Fibrosarcoma M-89	3			17
Leukemia K1964	3			0
Adenocarcinoma 755	3			11
Mammary carcinoma YMC	3			14
Reticulum cell sarcoma A-RCS	3			1
Lymphosarcoma P1798	2			0
Walker 256 carcinosarcoma	4			0
Osteosarcoma 124F	3			15

*Animals were injected ip daily for 10 days, beginning 1 day after tumor transplant.
†Nos. in parentheses = percent of long-term survivors.
‡Three-dimensional measurements of sc transplanted tumors were taken 20 days after transplant with the exception of the Walker 256 carcinosarcoma which was measured 10 days after transplantation.

lium nitrate in some solid rodent tumors and the increased uptake of its radionuclide by human tumors, a phase I study of gallium nitrate has been initiated at the NCI. Preliminary results in eight patients with various tumors, including four patients with melanoma, indicate no systemic toxicity at doses up to 180 mg/m² (approximately 4 mg/kg). Preclinical toxicology studies suggest that renal and hepatic damage might be expected, and these organ systems will be carefully monitored as patients are entered at the higher dose levels.

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TABLE 10.—Effect of anion substitution on antitumor activity of group IIIa metal salts against ascites Walker 256 carcinosarcoma in rats*

Salt	Optimal antitumor dose (mg/kg)	Increase in MST (% of controls)	No. of long-term survivors/ No. of animals treated†
Aluminum nitrate	150	300	5/12 (42)
$AlCl_3 \cdot 6H_2O$	129	550	4/12 (33)
$Al_2(SO_4)_3 \cdot 18H_2O$	89	200	3/12 (25)
Gallium nitrate	60	138	4/12 (33)
$GaCl_3$	25	180	1/12 (9)
$Ga_2(SO_4)_3 \cdot 18H_2O$	45	138	2/12 (17)
Indium nitrate	1.0	124	0/18 (0)
$InCl_3$	1.7	50	0/12 (0)
$In_2(SO_4)_3 \cdot 5H_2O$	0.8	27	0/12 (0)
$In(OOCCH_3)_3$	1.6	32	0/12 (0)
Thallium chloride	3.0	769	3/12 (25)
$Tl(OOCCH_3)_3$	3.9	427	2/12 (17)

*Rats were given daily ip injections of the appropriate salt beginning 1 day after tumor transplantation. At least 3 doses of each salt $\leq$ the LD10 for that salt were tested.

†Animals surviving 4 months with no histologic evidence of tumor are termed "long-term survivors." Nos. in parentheses = percent of long-term survivors.

TABLE 11.—Percent inhibition of the DNA polymerases of Walker 256 carcinosarcoma and of Rauscher murine leukemia virus by gallium nitrate and other antiviral agents*

Agent	Concentration (μg/ml)	% Inhibition of DNA polymerase from—				
		Walker 256				Rauscher murine leukemia virus
		A	B	C	D	
Gallium nitrate	50	0	40	0	30	59
Rifamycin SV	50	19	6	2	7	0
Rifamycin SV: *O*-*n*-octyloxime	25	62	31	66	40	62

*Assays were carried out in a total volume of 0.1 ml as described in the Materials and Methods section. The enzyme was preincubated with each drug 15 minutes at room temperature and then template and substrates were added. Incubations were carried out for 30 minutes, during which time the kinetics of polymerization were linear.

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Interaction of Rhodium(II) Carboxylates With Molecules of Biologic Importance[1]

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SUMMARY

Rhodium(II) acetate, propionate, and butyrate showed a considerable variation in their antitumor activity against Ehrlich ascites tumor cells in mice, with the butyrate complex being the most active. The three complexes markedly inhibited DNA synthesis of Ehrlich ascites tumor cells in vivo. Rhodium(II) butyrate was the most potent inhibitor followed by the propionate complex. One hour after administration, rhodium(II) propionate and butyrate induce more uridine-5-^{3}H incorporation into RNA than is seen in the controls. Equilibrium dialysis studies showed that rhodium(II) acetate-1-^{14}C binds to single-stranded DNA, poly-A, ribonuclease A, and bovine serum albumin but not to highly polymerized native calf thymus DNA, poly-G, or poly-C. In these cases binding occurred at the two axial positions of rhodium(II) acetate to a nitrogen donor in the ligands. The formation constants of the rhodium(II) acetate and propionate complexes with 5′-adenosine monophosphate were determined. The rhodium(II) propionate complex was more stable. Sedimentation and viscosity measurements of poly-A and poly-A/rhodium(II) acetate complexes indicate a high degree of intramolecular crosslinking in the rhodium(II) acetate/poly-A complex. The rhodium(II) carboxylate complexes were also found to be potent inhibitors of purified DNA polymerase I and RNA polymerase from *Escherichia coli.*

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Recently, we reported the results of a study of the effects of rhodium(II) acetate and propionate on the Ehrlich ascites and L1210 ascites tumors (1,2). Survival studies with Swiss mice bearing the Ehrlich ascites tumor and BDF_1 mice bearing the L1210 ascites tumor showed that rhodium(II) acetate and propionate gave rise to a significant increase in survival times. Rhodium(II) propionate was found to be a more potent drug than the acetate. In addition it was shown that both rhodium(II) complexes inhibit DNA and RNA synthesis in vitro, with DNA synthesis being inhibited to a greater extent. Rhodium(II) propionate was considerably more effective as an inhibitor than the acetate complex.

These findings prompted us to carry out more extensive studies using rhodium(II) acetate, propionate, and butyrate. In this paper we report the antitumor activity of the three rhodium(II) carboxylates at various dosages

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against Ehrlich ascites tumors carried in Swiss albino mice and the metabolic effects of these compounds on Ehrlich ascites tumor cells in vivo. The complexes formed between rhodium (II) carboxylates and several molecules of biologic importance have also been studied in order to understand the molecular interactions that produce the antitumor activity.

MATERIALS AND METHODS

Chemicals

Rhodium(II) acetate was purchased from Matthey Bishop, Inc. Rhodium(II) propionate and butyrate were synthesized by a method previously described (3).

5′-Adenosine monophosphate (5′-AMP) was obtained from Sigma Chemical Company. DNA polymerase I was obtained from Boehringer Mannheim (specific activity, 5650 units/mg). Calf thymus DNA was purchased from Sigma Chemical Company, as were unlabeled dATP, dCTP, dGTP, and dTTP. Thymidine-5′-triphosphate (methyl-^{3}H), tetrasodium salt, and deoxycytidine-5′-triphosphate (5-^{3}H) were purchased from Schwartz/Mann. The specific activities were 42 microcuries/nmol for the ^{3}H-TTP and 20 microcuries/nmol for the ^{3}H-CTP. Poly-dA · poly-dT and poly-dC · poly-dG templates were purchased from PL Biochemicals, Inc.

Poly-A (potassium salt) used in boundary sedimentation and viscosity experiments was purchased from Sigma Chemical Company.

Survival Studies

Female Swiss albino mice were each implanted intraperitoneally (ip) with 4×10^6 Ehrlich tumor cells. Twenty-four hours after implantation, all mice were randomly divided into groups of nine or ten mice. Rhodium(II) acetate and propionate were dissolved in 0.9% saline with gentle heat. Due to the insolubility of rhodium(II) butyrate in saline, the compound was initially dissolved in 95% ethanol and then diluted with 0.9% saline to a final concentration of 3% ethanol just before injection. Rhodium (II) acetate, propionate, and butyrate solutions were prepared fresh each time just before injection. Drug treatment was started 24 hours after implantation and injections were given ip once daily for 6 days. Control groups received ip injections of either saline or saline plus ethanol once daily for 6 days. Observations were continued for 60 days.

Measurement of LD10 of Rhodium(II) Carboxylates

The method of Skipper et al (4) was employed for this measurement. Briefly, five different groups of ten healthy Swiss albino mice per group received five different doses of the rhodium(II) carboxylates ip, and 9 days later, plots of mortality versus dose were made for each drug. The LD10 for each rhodium(II) complex was then determined from the corresponding graph.

Measurement of DNA Synthesis In Vivo

Mice bearing 5-day implants of Ehrlich tumor cells received rhodium(II) carboxylates or saline or saline plus ethanol. One positive control group received cytosine arabinoside[4] (15 mg/kg) which is a well-known inhibitor of DNA synthesis. Each group tested at a specified time point consisted of three mice. At various intervals after injection, thymidine-5-methyl-^{3}H (specific activity, 10 microcuries/mmol) was injected ip and metabolic utilization was allowed to proceed for 30 minutes. Tumor cells were harvested and washed four times with cold 5% TCA in the cold, and twice with cold 95% ethanol. The acid-insoluble precipitates were partially dried and each was resuspended in 2 ml of Protosol (New England Nuclear Corp.) and then incubated at 55° C.

Samples were counted in 10 ml of toluene scintillation fluid in a Packard Model 3380 scintillation spectrometer.

Measurement of RNA Synthesis In Vivo

All experimental procedures employed were the same as those described for the experiment involving DNA synthesis except that uridine-5-^{3}H (specific activity, 10 microcuries/mmol) was used.

[4] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; ara-C.

Inhibition of DNA Polymerase I and RNA Polymerase From *Escherichia coli*

Enzyme inhibition studies using pure DNA polymerase I were carried out for 30 minutes at 37°C in a total volume of 0.5 ml. Each tube contained 80 mM potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$, and either 5.0 μg of activated calf thymus DNA or 10.0 μg of activated homopolymer template. In the case of inhibition using calf thymus DNA as the template, each tube contained 5 nmols each of dATP, dGTP, dCTP, and ^{3}H-dTTP (0.023 microcurie/nmol) and 2.32 units of polymerase. When activated poly-dA · poly-dT was used as the template, each tube contained 25 nmols each of dATP and ^{3}H-TTP (0.02 microcurie/nmol) and 4.64 units of polymerase. When the template was activated poly-dC · poly-dG, each tube contained 25 nmols each of dGTP and ^{3}H-dCTP (0.02 microcurie/nmol) and 4.64 units of polymerase.

Templates were activated with DNA polymerase I in the presence of 1 mg/ml of BSA, 0.01 M $MgCl_2$, and 0.01 M tris buffer (pH 8.6). One mg/ml of template was incubated with DNA polymerase I for 15 minutes at 37° C. The DNA polymerase was inactivated by heating at 80° C for 5 minutes.

Boundary Sedimentation

All sedimentation experiments were done using a Beckman Model E analytical ultracentrifuge. The sedimentation coefficients were obtained at 25° C using 0.1 M potassium phosphate buffer (pH 7.5) as solvent. The poly-A solution was scanned at 289 nm, and the rhodium(II) carboxylate/polymer complex was scanned at 304 nm.

Binding Studies

Rhodium(II) acetate-1-^{14}C was dissolved in 0.01 M tris-HCl (pH 7.2) and 0.02 M NaCl to give a final concentration of 10^{-4} M. Each dialysis bag contained 0.5 ml of each biopolymer (2 mg/ml). The dialyses were carried out at 37°C. At the indicated times, each dialysis bag was taken out and a 0.1-ml aliquot was mixed with 5 ml of Aquasol and counted.

Viscosity Measurements

Viscosity data were obtained from an Ubbelohde viscometer immersed in a 25° C constant-temperature bath. All solutions to be measured were filtered through a medium-porosity glass funnel to remove dust. The rhodium(II) acetate/poly-A complex was not filtered. Rather, the solutions used to make the complex were filtered, and the complex was then made from them. Before each measurement, the viscometer was cleaned with a Chromerge solution, rinsed thoroughly with deionized water and acetone, and allowed to dry.

Formation Constants From Spectral Data

A spectrophotometric method was used to determine the stepwise formation constants. Because of the blue-green to pink color change produced by the complexation reaction, the extent to which the reaction has proceeded can be monitored by scanning the visible region. A Cary model 14 spectrophotometer and 10-cm pathlength cells were used in this study. All formation constants were obtained at 22° C in 0.1 M potassium phosphate buffer (pH 7.5). The titration procedure involved the addition of 1×10^{-2} M 5'-AMP to a solution containing 4.0×10^{-4} M rhodium(II) carboxylate. After each addition of ligand, the absorbance was measured in the region from 590 to 530 nm. All titrations were effected using a Manostat digital pipette. The formation constants were calculated using a rigorous least squares method similar to that developed by Wentworth (5).

RESULTS AND DISCUSSION

The LD10s for rhodium(II) acetate, propionate, and butyrate were each determined using Swiss albino mice prior to the survival studies. The LD10s for the acetate, propionate, and butyrate complexes were found to be 19, 2.7, and 0.7 mg/kg respectively.

The results of the survival studies with mice bearing tumors are shown in table 1. A dose-response curve was constructed from the data in table 1 and is shown in figure 1. The therapeutic indices (LD10/ILS40) shown in table 1 were calculated in order to compare the relative potencies of each rhodium(II) complex.

As can be seen in table 1 and figure 1, the largest increases in lifespans (ILS) were achieved when the rhodium(II) carboxylates were given close to the LD10 dosages. Doses greater than the LD10 apparently resulted in reduced lifespans due to toxic effects. Of the three rhodium(II) complexes tested, rhodium (II) butyrate gave the largest percent ILS (197%) in mice bearing the Ehrlich ascites tumor. However, the therapeutic indices of rhodium(II) butyrate and propionate were almost the same: 4.7 and 4.2 respectively. Both compounds were found to be considerably more potent than rhodium(II) acetate as antitumor agents.

There are several possible explanations for this observation. One is that the higher lipophilicity of rhodium(II) butyrate as compared to the other two rhodium(II) carboxylates enables a higher percentage of the drug to be transported into the tumor cells. Another explanation is that rhodium(II) butyrate forms a thermodynamically more stable complex with the biologic molecules involved. The individual kinetic and thermodynamic stabilities of the rhodium(II) carboxylates are also factors which could cause differences in antitumor activity. Present information indicates that the increased antitumor activity of the higher molecular weight rhodium(II) carboxylates is a result of some combination of the factors mentioned.

We have previously reported that both rhodium(II) acetate and propionate inhibit DNA synthesis in vitro (2). In this study we measured the inhibitory effect of rhodium(II) propionate and butyrate on DNA synthesis involving Ehrlich cells in vivo at different times after drug administration. The results are shown in figure 2. Cytosine arabinoside (15 mg/kg) was used as a positive control in this study. Figure 2 shows that this drug inhibited thymidine incorporation (presumably DNA synthesis) into DNA by more than 90% at 1 hour after drug administration. After this time, DNA synthesis increased gradually and reached approximately 90% of the control level 24 hours after drug administration. Neither rhodium(II) propionate (2 mg/kg) nor butyrate (0.6 mg/kg) showed appreciable inhibition of DNA synthesis 1 hour after the drug was given. However, the degree of inhibition increased as a function of time to greater than 90% at 24 hours. It is interesting to note that certain platinum(II) pyridine complexes also inhibit DNA synthesis of Ehrlich tumor cells in vivo over a 24-hour period after drug administration. (6).

Rather unusual results were obtained from

TABLE 1.—Survival studies with Swiss albino mice bearing Ehrlich ascites tumors

	Doses (mols/kg/day × 6 days)	%ILS	($LD10^{TI}$/ ILS40)
Control	Saline	—	—
Rhodium(II) acetate	6.78×10^{-4}	78	
	3.39×10^{-4}	88	1.8
	2.26×10^{-4}	44	
Rhodium(II) propionate	8.04×10^{-4}	—	
	5.02×10^{-4}	143	4.2
	2.01×10^{-4}	149	
	1.00×10^{-4}	17	
Control	Saline + ethanol	—	—
Rhodium(II) butyrate	3.61×10^{-4}	—	
	1.80×10^{-4}	132	
	1.08×10^{-4}	197	4.7
	5.40×10^{-7}	173	
	1.80×10^{-7}	3	

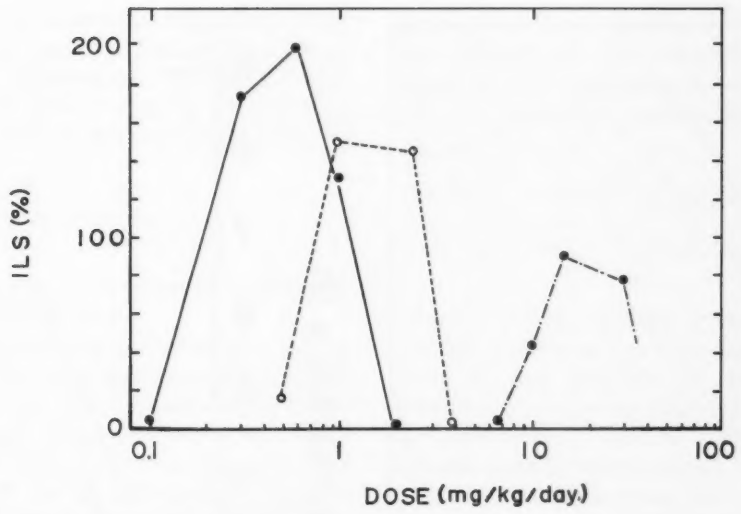


FIGURE 1.—Dose-response curve. Therapeutic indices were obtained from these curves. ●——●——● = Rhodium(II) butyrate; ○----○----○ = rhodium(II) propionate; ●—.—.●—.—.● = rhodium(II) acetate.

the RNA synthesis studies as shown in figure 3. Rhodium(II) propionate and butyrate both exhibited a slight inhibition of RNA synthesis 1 hour after the drug was given. However, after 1 hour there was a continuous increase in uridine-5-^{3}H incorporation in the drug-treated groups which was up to 1.5 times that noted in the controls at approximately 11 hours after drug was given. After this time there was a gradual decrease in incorporation; however, the amount was still greater than in the control groups 24 hours after drug. It is not known why the administration of rhodium(II) complexes causes a higher rate of uridine-5-^{3}H incorporation into RNA than is noted in the control groups. However, the following two possibilities may be conjectured: (*a*) the rhodium(II) carboxylates may actually stimulate the RNA synthesis of Ehrlich tumor cells in vivo; and (*b*) the rhodium(II) carboxylates may interfere with the uridine (or UTP) precursor pools so that more uridine-5-^{3}H is incorporated into the acid-insoluble nucleic acid.

In order to determine to what type of macromolecules rhodium(II) acetate binds we carried out equilibrium dialysis studies using rhodium(II) acetate-1-^{14}C with native calf

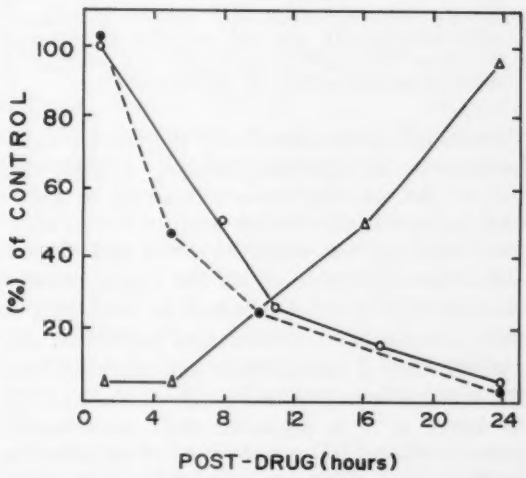


FIGURE 2.—In vivo inhibition of DNA synthesis by rhodium(II) carboxylates. ○——○——○ = Rhodium(II) butyrate (0.6 mg/kg); ●----●----● = rhodium(II) propionate (2 mg/kg); -----△-----△ -----△ = cytosine arabinoside (15 mg/kg). A 30-min labeling time of thymidine-5-methyl-^{3}H (specific activity, 10 microcuries/mmol) was not included in post-drug intervals. Each point represents the average of three mice per group.

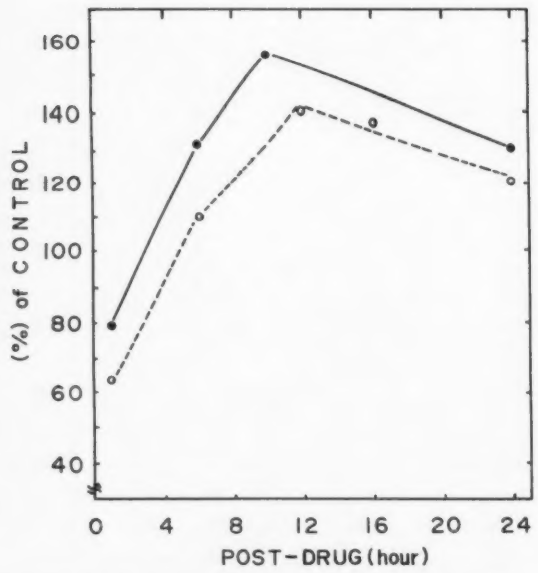


FIGURE 3.—In vivo inhibition of RNA synthesis by rhodium(II) carboxylates. ●——●——● = Rhodium(II) butyrate (0.6 mg/kg); ○----○----○ = rhodium(II) propionate (2 mg/kg). A 30-min labeling time of uridine-5-^{3}H (specific activity, 10 microcuries/mmol) was not included in post-drug intervals. Each point represents the average of separate determinations on three mice.

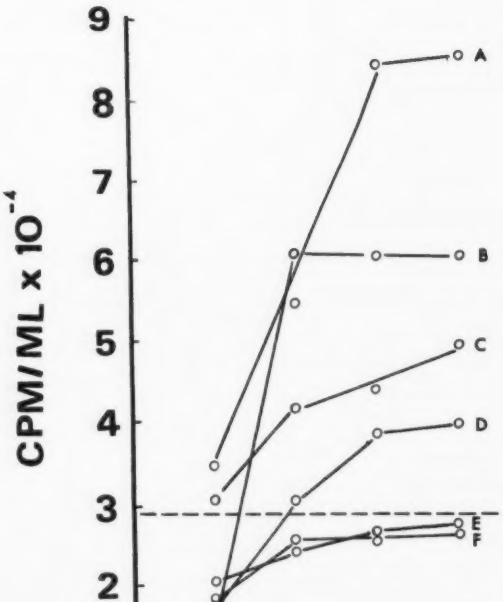
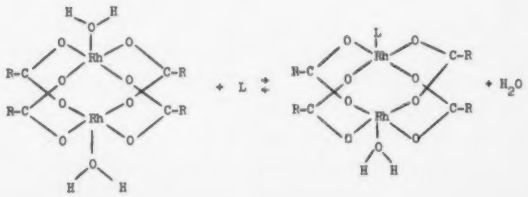


FIGURE 4.—Binding of rhodium(II) acetate-1-^{14}C to several macromolecules as determined by equilibrium dialysis. A = poly-A; B = ribonuclease A; C = denatured RNA; D = albumin; E = poly-G = native DNA; F = poly-C.

thymus DNA, denatured calf thymus DNA, ribonuclease A, albumin, poly-A, poly-G, and poly-C. As seen in figure 4, binding to native calf thymus DNA did not seem to occur. However, binding was observed when calf thymus DNA was denatured. With the homopolymers, rhodium(II) acetate appeared to bind only to poly-A under the experimental conditions employed. Also, if comparisons are made between the binding to poly-A and single-stranded DNA in figure 4, it is apparent that considerably more rhodium(II) acetate-1-^{14}C was taken up by the poly-A. This again indicates that it was only the adenine moiety of single-stranded DNA that bonded to the acetate complex. Figure 4 also shows that binding occurred to the proteins, ribonuclease A and bovine serum albumin.

From our knowledge of the chemistry of rhodium(II) carboxylates (7–9) there are two ways in which the rhodium(II) carboxylates could bind to the biopolymers. Binding could take place at the two axial positions of the rhodium(II) carboxylates to an electron pair donor atom of a ligand L:



With macromolecules, both axial positions could be involved in the bonding through interstrand or intrastrand crosslinking. The second possibility for the binding would be the substitution for one or more of the carboxylate ions by a ligand:

$$Rh_2(RCOO)_4 + L \rightleftarrows Rh_2(RCOO)_3L + RCOO^-$$

This reaction is known to occur at a slow rate when L is a carboxylic acid.

When rhodium(II) acetate binds to a donor-type ligand at the axial positions, the color of the complex is found to depend on the donor atom of the ligand. Those adducts in which the oxygen atom of the ligand was bonded to the rhodium were green or blue-green; those involving nitrogen donors were pink, rose-red, or violet; and those involving sulfur were violet or orange (7–9).

In all cases where binding occurred with the biopolymers, there was a color change of the rhodium(II) acetate complex from blue-green to pink. This indicates strongly that binding was occurring at one or both of the axial positions of rhodium(II) acetate through a nitrogen donor of the ligand. In cases where bonding did not occur no color change was observed. Also, if hydrochloric acid is added to a solution containing rhodium(II) acetate and 5′-AMP, the color of the solution is blue below pH 4 (where the nitrogen donors are protonated) and pink above pH 4. This color change is rapid and reversible on adjustment of the pH. These experiments produce strong evidence for axial binding (adduct formation) through a nitrogen donor. Of course this does not prove that this is the type of reaction that is producing the antitumor activity.

The disparate effects seen with the rhodium (II) carboxylates with respect to their antitumor properties could be due to differences in the thermodynamic stability of the complexes they form (adducts) with biologic molecules. If, for example, rhodium(II) propionate forms a more stable complex with 5′-AMP than does the acetate, it should, therefore, be a better inhibitor of processes involving 5′-AMP. This argument can be extrapolated to 5′-ADP, 5′-ATP, and finally, nucleic acids which contain exposed adenine residues. For this reason, we have determined the formation constants of rhodium(II) acetate and propionate complexes with 5′-AMP. The reactions studied were:

$$Rh_2X_4\cdot 2H_2O + 5'\text{-AMP} \overset{k_1}{\rightleftharpoons} Rh_2X_4\cdot(H_2O)(5'\text{-AMP}) + H_2O$$

$$Rh_2X_4\cdot(H_2O)(5'\text{-AMP}) + 5'\text{-AMP} \overset{k_2}{\rightleftharpoons} Rh_2X_4\cdot 2(5'\text{-AMP}) + H_2O$$

where X was the acetate or propionate ion. As can be seen from the formation constants in table 2, the stability of the rhodium(II) propionate complexes is about double that of the rhodium(II) acetate complexes. The increased stability of the rhodium(II) propionate complexes could account for part of the increase in toxicity, anticancer activity, and enzyme inhibition observed for the propionate complex.

In another study we determined the rate of exchange of the trifluoroacetate ion for one carboxylate ion of rhodium(II) acetate, propionate, and butyrate. Each of the rhodium(II) carboxylates was dissolved in trifluoroacetic acid and the rate of exchange was followed by the appearance of the free carboxylic acid. A Varian HA-100 nuclear magnetic resonance spectrometer was used to monitor the reaction. The pseudo-first order rate constants at 20° C were 1.4, 1.5, and 1.3 × 10^{-4} sec^{-1} for the acetate, propionate, and butyrate respectively. Under the experimental conditions employed all three exchange reactions occurred at essentially the same rate. Therefore, the vast differences in biologic activity of these rhodium(II) carboxylates cannot be accounted for in terms of the differences in the exchange rate.

In order to investigate the nature of the rhodium(II) acetate/poly-A complex we carried out hydrodynamic studies on the system. The

TABLE 2.—Formation constants for rhodium(II) carboxylate complexes of 5′-AMP

Rhodium(II) carboxylate	K_1	K_2
Acetate	1893 ± 99	202 ± 14
Propionate	4235 ± 267	344 ± 20

results of this study are shown in table 3. Striking effects upon the hydrodynamic behavior of poly-A in the presence and absence of rhodium(II) acetate are clearly demonstrated. In the case of the data at the higher poly-A concentration, a ratio of approximately one rhodium(II) acetate molecule for each 4.4 nucleotide residues produced a 62% decrease in the reduced viscosity from the value in the absence of the metal compound, while concomitantly causing a 92% increase in the observed sedimentation coefficient. With one rhodium(II) acetate moiety for every 0.45 nucleotide residues, a 71% decrease in the reduced viscosity was obtained. Attempts to measure a sedimentation coefficient for this complex led to very diffusely sedimenting boundaries from which reliable sedimentation data were not obtainable. However, the half-height of the boundary was clearly travelling much more rapidly than would the poly-A alone. Essentially no absorption was observed centripetal to the moving boundary throughout the sedimentation experiments reported in table 3, indicating that virtually all the rhodium(II) acetate was bound and was sedimenting with the polymer.

There are three hypotheses which could be advanced to explain the effect of rhodium(II) acetate upon the hydrodynamic properties of poly-A: (*a*) the polymer remains essentially in the same hydrodynamic conformation it has in the absence of the metal compound, with the effect upon the reduced viscosity and sedimentation coefficient primarily due to the increased molecular weight due to the bound rhodium compound; (*b*) the polymer could be intermolecularly crosslinked through the binding of the two axial positions to adenylate residues in different molecules of poly-A; and (*c*) intramolecular crosslinking, in which both axial positions are bound to adenylate residues within a single poly-A chain, takes place extensively. Only the third of these is consistent with the data in table 3.

If one rhodium(II) acetate moiety is bound per each 4.4 adenylate residues, the molecular weight of the complex is 1.27 times that of the poly-A alone. If the rhodium compound had no effect upon the partial specific volume and frictional coefficient of the molecule, the sedimentation coefficient would be increased only by this factor (10). It is far more likely that the partial specific volume would be decreased and that the frictional coefficient (if no intermolecular or intramolecular binding takes place) would be slightly increased; both effects would decrease the observed sedimentation coefficient. Considering also the effect of a molecular weight increase alone, the reduced viscosity should result in a slight increase; the interpretation of the changes as predominantly due to a molecular weight increase can be convincingly ruled out.

The intermolecular crosslinking to yield large aggregates could indeed cause a substantial increase in the sedimentation coefficient, but this should also result in an increase in the reduced viscosity. This interpretation appears untenable.

The intramolecular crosslinking hypothesis, however, is entirely consistent with the data. The formation of multiple "hairpin" structures would drastically reduce the hydrodynamic volume of the polymer, causing a marked decrease in the frictional coefficient and therefore increases in the sedimentation coefficient. The viscosity of the hydrodynamically less extended complex would be fully expected to decrease in qualitative accordance with the observed results.

TABLE 3.—Results of sedimentation and viscosity measurements of poly-A and poly-A/rhodium(II) acetate complexes in 0.1 M potassium phosphate buffer (pH 7.5) at 25°C

Poly-A concentration (mols of nucleotide/liter)	Rhodium(II) acetate concentration (M)	Reduced viscosity (dl/g)	Observed sedimentation coefficient (Svedberg units)
2.2×10^{-3}	0	1.42	5.9
2.2×10^{-3}	5.0×10^{-4}	0.54	11.3
4.5×10^{-4}	0	0.581	—
4.5×10^{-4}	1.0×10^{-3}	0.167	—

Such a drastic effect upon the conformation of a polymer in solution is not expected to be without biologic effects. One very interesting possible target for this particular mode of rhodium(II) acetate interaction would be the poly-A chain which is well known to be affixed to the 3′ end of messenger RNAs in the eukaryotic cell.

In vivo rhodium(II) carboxylates are potent inhibitors of DNA synthesis, yet they seem to stimulate RNA synthesis. In order to separate effects of the drugs on polymerase enzyme systems from those effects due to other factors, ie, cell permeability, inhibition of other enzymes, etc, inhibition studies were conducted involving purified DNA polymerase I and RNA polymerase from *E. coli*. Incubation times of these inhibition studies were equal to or less than ½ hour. Therefore, the results should indicate short-term effects exerted on the enzyme system by the drug, and most effects would be due to the more rapid axial-type binding rather than exchange for a carboxylate ion. It can be seen from table 4 that the rhodium(II) carboxylates tested do indeed inhibit both DNA polymerase I and RNA polymerase. Three possible modes of action are likely: (*a*) inhibition due to action of the drug on the enzyme, either on the active site or on the surface of the enzyme; (*b*) inhibition due to binding of the substrate pool, particularly those molecules containing adenine; and (*c*) inhibition due to binding of the drug to single-stranded regions of the template, and in particular, to adenine residues of the template. Of course, inhibitions could be due to some combination of these factors.

In general, the DNA polymerase system seems more sensitive to inhibition by rhodium (II) carboxylates than does the RNA polymerase system. This effect is especially evident at lower concentrations of the inhibitor. This could be due, to some extent, to the nature of the templates in each of the systems. Since DNA polymerase I acts on activated (denatured) template whereas the RNA polymerase requires native calf thymus DNA as template, the increased inhibition in the former system could be due to the inhibitor binding to the single-stranded regions of the template.

The most striking feature of these studies is the order of effectiveness of the inhibitors, ie, acetate < propionate < butyrate. This rules out differences in cell permeability as the only causative factor of the disparate effect seen with these inhibitors.

Our earlier binding studies, using equilibrium dialysis, showed that rhodium(II) acetate binds to poly-A but not to poly-C and poly-G. Using this information, we designed an experiment to distinguish between inhibitory effects due to binding of the inhibitor to template and/or substrate and those effects produced by binding to the enzyme. Two systems were employed: one in which the template and substrate could bind to rhodium(II) carboxylates (poly-dA, poly-dT; dATP, dTTP) and one in which binding to template or substrate does not occur (poly-dC, poly-dG; dCTP, dGTP). The enzyme

TABLE 4.—Inhibition of DNA polymerase I and RNA polymerase from *E. coli*

Concentration (M × 10^5)	Percent inhibition of polymerases by rhodium(II)—					
	Acetate		Propionate		Butyrate	
	RNA	DNA	RNA	DNA	RNA	DNA
40.0	95	92	100	99	—	—
20.0	63	91	94	98	—	—
10.0	—	—	—	—	100	—
8.0	30	71	80	95	100	100
6.0	—	—	52	—	—	—
5.0	—	—	—	—	100	100
4.0	8	53	28	94	100	—
3.0	—	—	—	—	95	95
2.0	—	—	—	—	75	—
1.0	0	18	9	86	22	94
0.5	—	—	—	63	—	92
0.1	—	0	—	0	—	41

TABLE 5.—Inhibition of DNA polymerase by rhodium(II) carboxylates using poly-dA • poly-dT and poly-dC • poly-dG as templates

Template	Percent inhibition of polymerase by rhodium(II)— Acetate (4×10^{-5} M)	Propionate (4×10^{-6} M)	Butyrate (4×10^{-6} M)
Poly-dA · poly-dT	83.9	33.1	20.0
Poly-dC · poly-dG	55.7	71.3	49.3

used was DNA polymerase I from *E. coli*. The results are shown in table 5.

The results seen with rhodium(II) acetate are those we expected to find. Rhodium(II) acetate inhibited the poly-dA · poly-dT system by 83.9%, where binding could occur to the template, substrate, and enzyme, and inhibited the poly-dC · poly-dG system by 55.7%, where binding could occur to the enzyme only. However, in the cases of rhodium(II) propionate and butyrate, the opposite results were obtained. These two complexes inhibited the poly-dC · poly-dG system considerably more. However, there is an explanation for these results which is not contrary to our original assumptions. The explanation involves the concentrations of all species present in the reaction mixture. The concentrations of rhodium(II) carboxylates were 4×10^{-5} M, 4×10^{-6} M, and 1×10^{-6} M for the acetate, propionate, and butyrate complexes respectively. The low concentrations of the butyrate and propionate complexes were necessary because they are such potent inhibitors. The concentrations of the substrates (dATP, dTTP, dCTP, and dGTP) were all 5×10^{-5} M. Also, the concentration of monomer units in the templates was 5×10^{-5} M. By comparing these concentrations it is easy to see that most of the rhodium(II) propionate and butyrate would be tied up with the dATP substrate leaving very little for binding to the enzyme. Because of the big excess of dATP there would still be enough of the free substrate for continued replication. In the case of the poly-dC · poly-dG system, all of the propionate and butyrate complex binds to the enzyme producing a high inhibition. More work is needed to be sure of this interpretation.

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Review of the Current Clinical Status of Platinum Coordination Complexes in Cancer Chemotherapy [1]

Jeffrey A. Gottlieb and Benjamin Drewinko [2,3]

SUMMARY

During the past 3 years, eight hospitals and one cooperative study group have reported their initial clinical results with *cis*-dichlorodiammineplatinum (II). The most popular clinical schedule was 15–25 mg/m²/day for 5 days repeated every 3–4 weeks. Almost all patients had extremely advanced disease. Of 323 patients in whom response could be evaluated, there were 12 complete responses, 25 partial responses (>50% decrease in tumor size), and 23 improvements (<50% decrease in tumor size) for a 19% overall response rate. The tumor most sensitive to *cis*-dichlorodiammineplatinum (II) was testicular carcinoma in which seven complete responses, three partial responses, and three improvements were observed in 16 patients treated at Roswell Park Memorial Institute. Other sensitive tumors were lymphoma (63% response and improvements), squamous cell carcinoma of the head and neck (41% response and improvements), and ovarian carcinoma (40% response and improvements). Complete responses were also seen in one patient with thyroid carcinoma and two with bladder carcinoma, while partial remissions were recorded in two patients with breast carcinoma and one patient each with acute myelogenous leukemia, endometrial carcinoma, renal carcinoma, malignant thymoma, neuroblastoma, adenocarcinoma of the lung, and an undifferentiated tumor of unknown origin. Five major types of toxicity were encountered: gastrointestinal, hematopoietic, immunosuppressive, otologic, and renal, with the last two generally the most serious. Serial audiometry testing can generally warn of the otologic toxicity and thus prevent permanent acoustic damage. Renal toxicity, which is similar to that seen with heavy-metal poisoning, appears to be dose related, cumulative, and only partly reversible, thus, severely limiting the repeated administration of *cis*-dichlorodiammineplatinum (II). Recent laboratory studies suggest that combination chemotherapy with this drug may be rewarding. Studies of this nature should be pursued along with attempts to find more effective less toxic platinum compounds.

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This paper emphasizes the current clinical status of platinum coordination complexes in chemotherapy as now understood by investigational clinical oncologists. It deals mainly with published reports and hopefully reflects the opinions of physicians who follow the medical literature carefully and have perhaps used the drug in a small select group of patients. While the main goal of the paper is to present the current clinical status of the platinum coordination complexes, emphasis is also given to problems faced in the clinic where help is needed from those who work in the field of medicinal chemistry. The only metal drug that has been used in published clinical studies is *cis*-dichlorodiammineplatinum(II).[4] A few other platinum compounds are now undergoing some early clinical studies (1,2) but reports of these trials have not yet reached the literature and are therefore not included in this review.

CLINICAL EFFICACY

Table 1 lists the reports that form the basis of this review. While none of the response rates shown are very encouraging, it must be emphasized that most of these studies were of a phase I nature, ie, the physicians were trying to learn how to use the drug, often in terminally ill patients. The diagnoses included in the studies listed must also be known. Wiltshaw and Carr's 37% response rate is from a study confined to patients with ovarian carcinoma (3), a relatively responsive tumor, while Dr. Kovach and his colleagues at the Mayo Clinic, who recorded a response rate of 4% (4), treated only colorectal tumors, obviously a very resistant cancer. These studies are important in that they explored ways to use the drug. It was shown that daily injections for 5 days should not exceed 20–25 mg/m^2 (5, 6), the best weekly dose is probably 40–50 mg/m^2, and the best monthly dose is 75–100 mg/m^2 (5, 7). The response rates by all the different schedules thus far appear roughly the same. The toxic effects of *cis*-dichlorodiammineplatinum(II) were also learned during these studies and are discussed below.

Table 2 shows that the percentage of patients with complete responses was small, only 4%. Only an additional 8% of the population had partial remissions, and a few more improved in some way with little tumor shrinkage. In total, however, only 19% of all the patients receiving the platinum compound in these studies improved in any way, and 81% failed to have any beneficial effect. While several drugs, such as mithramycin (8),[5] that have subsequently been proven to be quite effective for some specific tumors have had no greater general activity at the phase I level, other recently introduced clinical compounds, such as adriamycin,[6] have shown a much higher level of efficacy in studies of the same type, as high as 60% using the same response criteria (9).

It is only by analyzing the responses according to diagnosis that it can be seen how data derived from clinical trials of this nature may be useful. Thus, as shown in table 3, of the 16 patients with testicular carcinoma, seven had complete responses, three had partial responses, three improved, and only three failed to have some benefit from the drug (5,7). In lymphoma, including both Hodgkin's disease and non-Hodgkin's lymphoma, the response rate is also high (6,7,10),[7] and in two other tumors, squamous cell carcinoma of the head and neck (11) and ovarian carcinoma (3), response rates of 40% or greater have been recorded, suggesting that *cis*-dichlorodiammineplatinum(II) clearly has possible chemotherapeutic potential. It is important to emphasize that testicular carcinomas and lymphomas are among the most sensitive tumors to cancer chemotherapy, and indeed the tumors in which this drug is active are also quite similar to those responsive to alkylating agents (12). There are, however, studies which suggest that this drug's activity cannot be explained solely by an alkylating agent-like effect on nucleic acids (13,14).

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[4] *Cis*-dichlorodiammineplatinum (II): NSC-119875; CAS reg. No. 15663-27-1.

[5] Mithramycin: NSC-24559; CAS reg. No. 18378-89-7.

[6] Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

[7] Rossof AH, and Coltman C, Jr. Personal communication.

TABLE 1.—Overall clinical results of studies with *cis*-dichlorodiammineplatinum (II)

Investigators	Ref. No.	Institute	No. of— Patients	No. of— Responses	Response rate (%)
Wiltshaw and Carr	3	Royal Marsden Hospital	19	7	37
Higby et al	5,15,16	Roswell Park Memorial Institute	50	17	34
Lippman et al	11	Memorial Hospital for Cancer and Allied Diseases	21	7	33
Hill et al	10	Wadley Institutes	63	13	21
Rossof and Coltman	7	Rush-Presbyterian-St. Luke's Hospital	31	5	16
Rossof et al	(*see* footnote 7)	Wilford Hall US Air Force Medical Center	21	3	14
DeConti et al	17	Yale University	10	1	10
Talley et al	6	Southwest Oncology Group	57	5	9
Kovach et al	4	Mayo Clinic	51	2	4
Total			323	60	19

TABLE 2.—Extent of clinical responses in studies with *cis*-dichlorodiammineplatinum (II)*

	No. of patients	
Evaluable patients	323	
Complete responses	12 (4%)	19%
Partial responses	25 (8%)	
Improvements	23 (7%)	
Failed therapy	263 (81%)	

*From studies shown in table 1.

Table 3 also lists the other tumors in which activity was observed. These results indicate that the drug affected a few patients with bladder cancer (5,15,16), thyroid cancer (5, 15,16), breast carcinoma (7,10), and to a lesser extent the other diseases listed. Further studies using this drug at the phase II level in these tumor types might be indicated. What is not given here, and in fact, what is not always included in the literature, is the number of patients treated. Thus, it can be seen that there are three patients listed as improved with colon carcinoma (4,10), but for this particular tumor more than 50 patients were treated (4). Therefore, further exploration is not warranted in this disease, at least not with *cis*-dichlorodiammineplatinum(II) as a single compound, but studies with combination therapy might still be useful (a point covered in greater detail below). On the other hand, the number of patients with acute myelogenous leukemia treated is relatively small, and the two minimal responses seen (10,17) might warrant further investigation. In most of the other tumor types, the literature is not clear as to how many patients failed to respond.

Table 4 shows the responses in testicular carcinoma according to the cell type. This table indicates that all of the major types of testicular carcinoma treated (no teratocarcinomas have thus far been reported) seem equally responsive to this drug (5), an encouraging trend which hopefully will be confirmed by larger studies. The durations of the responses, which range from 1 to 10 months, are also shown. In almost all patients, either drug resistance or drug toxicity finally necessitated discontinuing the therapy. While these responses are not very long, for some patients they were very significant, and it must be emphasized that

TABLE 3.—Responses according to diagnosis from studies with *cis*-dichlorodiammineplatinum (II)*

Diagnosis	Patients	No. of— Complete responses†	Partial responses‡	Improvements§	Total response rate (%)
Testicular carcinoma	16	7	3	3	81
Lymphoma	16	2	7	1	63
Squamous cell carcinoma of the head and neck	17	0	1	6	41
Ovarian carcinoma	20	0	5	3	40

*From studies shown in table 1.

†Other complete responses: bladder carcinoma (2); thyroid carcinoma (1).

‡≥50% tumor reduction. Other partial responses: breast carcinoma (2); acute myelogenous leukemia, endometrial carcinoma, renal carcinoma, thymoma, neuroblastoma, lung adenocarcinoma, unknown undifferentiated primary (1 each).

§ <50% tumor reduction, significant subjective improvement, or mixed response. Other improvements: colon (3); multiple myeloma, breast carcinoma, acute myelogenous leukemia, lung carcinoma, prostatic carcinoma, unknown undifferentiated primary, undifferentiated sarcoma (1 each).

TABLE 4.—Responses to *cis*-dichlorodiammineplatinum (II) in testicular carcinoma*

Diagnosis	No. of— Patients	Complete responses	Partial responses	Improvements
Embryonal cell carcinoma	8	2	2	2
Embryonal cell, mixed	4	1	1	1
Choriocarcinoma	2	2	—	—
Seminoma	2	2	—	—
Totals	16	7	3	3
Durations of responses (mos)		10,4,3,3,2, 1,1	5,4,3	3,2,1

*Data of Higby et al (5) and Rossof et al (7).

almost all the patients who responded to the platinum drug had already failed more conventional therapy, usually radiotherapy and at least one other drug (mainly actinomycin D [NSC-3053]) (5). It is interesting to note that in animal toxicology studies, a loss of sperm production and testicular atrophy were observed (18), which might have been an indicator that platinum would affect testicular tumors. Combination regimens utilizing plati-

num with other drugs known to be effective in testicular tumors are now being tested at several institutions (19), and the results of these studies are eagerly awaited.

There is currently a considerable amount of laboratory data suggesting that *cis*-dichlorodiammineplatinum(II) is synergistic or at least additive in its effects with other drugs (20,21). Unfortunately, most of the data suggesting that this synergism exists are from experiments where both the tumor and the drug were given intraperitoneally. Because the drug concentration achieved by this route of administration and the rate of drug absorption may vary considerably from the clinical situation, the validity of these studies must be suspect until their relevance to the clinical situation has been proved. Nevertheless, in a study from the Mayo Clinic involving 51 patients with advanced colorectal carcinoma, only two patients had even a mixed response to the drug (4). In contrast, Ellerby and his colleagues at the University of Wisconsin were able to produce three partial remissions (two of which were still continuing at the time of publication, and one of which occurred in a "5-FU-resistant"[8] patient) in the eight patients they treated with *cis*-dichlorodiammineplatinum(II) plus 5-FU, a drug known to be effective in colon carcinoma (22). They hoped to observe a synergistic or additive effect with the combination. Since a response rate of three of eight patients has been seen using 5-FU alone (12), this series will need to be extended before a clear role for the platinum compound in combination regimens for colorectal carcinoma can be established.

TOXICITY

The major types of clinical toxic effects of *cis*-dichlorodiammineplatinum(II) are gastrointestinal, hematopoietic, immunosuppressive, otologic, and renal (6–8,11,15). The first three of these side effects are relatively mild. Gastroenteric toxicity consists mainly of nausea and vomiting lasting from 4 to 6 hours and occasionally in some sensitive patients even up to 1 week (6,7,11). Anorexia unfortunately may be cumulative, and some patients develop less and less appetite even while their tumors are shrinking (5,15). Hematologic toxicity appears to be excessive at doses exceeding 100 mg/m² given monthly or 24 mg/m² given daily for 5 days, and is expressed mostly as leukopenia which is maximal about 4 weeks after therapy and occasionally by associated mild thrombocytopenia (5–7). Basophilically stippled erythrocytes (indicating ribosomal injury) have not been observed in the bone marrows of patients treated with this drug (7). Since most of the toxicity of *cis*-dichlorodiammineplatinum(II) is similar to that produced by other heavy metals, such as lead where stippled red blood cells are regularly observed, it is puzzling that we have not yet observed this clinical phenomenon with the platinum drug. The importance of the drug's immunosuppressive properties is not yet well understood in man, but this effect is being investigated extensively at the Wadley Institutes (23).

The otologic toxicity of platinum is quite serious because (*a*) it is almost always irreversible, (*b*) it is almost certainly cumulative, and (*c*) early in its course it is almost exclusively associated only with high-range hearing loss at the 4000–8000-Hz (cycles per second) level. This is important to emphasize because unless we audiologically test patients, they will not appreciate the fact that they are having this hearing loss. If the toxicity is ignored and the drug continues to be used, clinical hearing loss at the spoken-voice range of 1000–4000 Hz will occur (11). The point to be stressed here is that obviously unless we test patients to see if high-range hearing loss is occurring and then stop the drug in time, a long-lasting significant hearing loss will occur. At some doses that were subsequently shown to be lethal (because of their ultimate effects upon the hematopoietic and renal systems), clear-cut deafness preceded death (24). Tinnitus is also noted at high doses (24) and should be avoidable by audiologically testing patients to pick up the high-range hearing losses first, thus discovering that the point has been reached when the drug must be stopped (11).

While this toxicity is not very well understood (because it has not been seen very often with other antitumor drugs or with heavy-metal poisoning), it appears that it is the

[8] 5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil.

cochlea that is affected (11). This type of toxicity was not predicted in the first animal toxicology studies for the obvious reason that our regular toxicology screen does not have a good way of testing for hearing loss in animals. Now that the otologic toxicity has been seen in man, studies in laboratory animals do show cochlear dysfunction (25).

The most important toxic effect of *cis*-dichlorodiammineplatinum(II) occurs in the kidney. This eventually becomes irreversible in all patients who continue to be treated, at least with all the current regimens being investigated (5,11). There are some intermittent studies with the drug which seem to permit renal recovery, so that the cumulative effect is negligible. On the other hand, almost all long-term responders to the platinum drug have eventually required discontinuation of therapy because of this side effect (5). The toxicity presents itself as an elevation in the blood urea nitrogen (BUN) and serum creatinine levels as well as by a decreased creatinine clearance (6,7,11). Microscopically, the lesions appear to be renal tubular necrosis (7). This side effect was predicted by the animal toxicology studies (18) and appears to be similar to heavy-metal poisoning in man in general. It is here that clinicians need the greatest help from medicinal chemists, because (*a*) this side effect prohibits the long-term use of the drug even in patients who are responding (5), and (*b*) we are occasionally faced with the clinical situation where an infection requires antibiotics, which are also nephrotoxic. This problem does not arise frequently but is most significant when it does occur. A case of irreversible renal toxicity due to *cis*-dichlorodiammineplatinum(II) was recently reported. The BUN reached a maximal level approximately 2 weeks after the start of therapy and never returned to normal. Microscopically, areas of interstitial fibrosis with lymphocytic infiltrates, tubular destruction, and periglomerular fibrosis similar to that seen with lead poisoning were observed (26).

Thus far our efforts to alter renal toxicity have been unsuccessful. Spironolactone [9] was noted by Haddow and Lester to increase the excretion of mercury and therefore to decrease the incidence of mercury toxicity in rats (27). Drewinko et al (28) studied the effect of spironolactone on *cis*-dichlorodiammineplatinum(II) to see if this agent altered the efficacy of the platinum compound. These studies showed that the efficacy of the latter agent was unchanged, but in studies with spironolactone done in collaboration with Schabel at Southern Research Institute, no decrease in the incidence of renal toxicity in mice was observed.[10] A similar study done by Schaeppi et al with D-penicillamine,[11] the drug used in copper poisoning, also did not prevent laboratory animals from developing renal tubular necrosis.[12] This is clearly an area in which help from the medicinal chemist is needed, either by finding a way to prevent renal toxicity or by finding a compound which retains the clinical efficacy of *cis*-dichlorodiammineplatinum(II) but does not have this agent's side effects (29).

CONCLUSIONS

Platinum coordination complexes, a new class of antitumor drugs, have been added to the chemotherapeutic armamentarium available for the advanced cancer patient. *Cis*-dichlorodiammineplatinum(II) is the only metal compound with published clinical studies. The early results with the drug, while not overly encouraging, are not completely discouraging. Suggestive leads, particularly in the fields of testicular carcinoma, malignant lymphoma, squamous cell carcinoma of the head and neck, and ovarian carcinoma, have been uncovered, and there are some tantalizing preliminary results suggesting that combination studies may be more rewarding. Five types of toxic effects of this drug have been described: gastroenteric, hematopoietic, immunologic, otologic, and renal. The last two are dose-limiting and are currently holding back greater clinical exploitation. While clinicians are exploring new schedules and combina-

[9] Spironolactone: NSC-150399; CAS reg. No. 52-01-7; 17α-pregna-4-ene-21-carboxylic acid, 17-hydroxy-7α-mercapto-3-oxo-, γ-lactone, acetate.

[10] Schabel FM, Jr. Personal communication.

[11] D-Penicillamine: NSC-81549; valine, 3-mercapto-, D-.

[12] Schaeppi U, Fleischman RW, Phelan RS, et al. No decrease in toxicity by combined treatment with penicillamine in rhesus monkeys. Report to the Laboratory of Toxicology, Experimental Therapeutics, Division of Cancer Treatment, NCI.

tions in an attempt to discover more effective but less toxic ways of using this drug, it is hoped that basic research will provide a better metal complex to be used as an antitumor agent or better ways of handling the toxic properties of the agent we currently have.

ADDENDUM (April 20, 1975)

The complete report of the 2nd International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy (referred to in part by refs 3 and 19) has just been published and supplements several of the studies reported preliminarily in our monograph.

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Coordination Complexes of Platinum as Antitumor Agents [1]

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SUMMARY

Rosenberg and VanCamp first reported the bacteriostatic and antitumor properties of certain platinum coordination compounds. This pioneering work has led to the synthesis and testing of a large number of related platinum products and the clinical use of *cis*-dichlorodiammineplatinum (II). This drug has proven very efficacious against a wide variety of mouse tumors when employed as the sole chemotherapeutic agent and has also been combined successfully with other antitumor agents, such as cyclophosphamide and cytosine arabinoside. Unfortunately, *cis*-dichlorodiammineplatinum (II) has a low therapeutic index with renal toxicity being the principal limitation of its use. This has prompted the synthesis and testing of many related platinum compounds, of which *cis*-dichlorobiscyclopentylamine-platinum (II) and the "platinum blues" appear most promising. Experience in the synthesis, purification, and testing of such products will be discussed together with an identification of outstanding unsolved problems in this area.

[Cancer Chemother Rep 59:629–641, 1975]

In 1965, Rosenberg and VanCamp (1) first observed that certain coordination compounds of platinum inhibited bacterial replication. In 1969, they reported that these interesting products also had marked antitumor activity (2). The compound which has been studied most extensively is *cis*-dichlorodiammineplatinum (II) shown in figure 1. Probably what first focused attention on this drug was the discovery that it was remarkably effective in the treatment of a wide variety of animal tumors. These included leukemia L1210, Rauscher-induced mouse leukemia MCDV 12, Gardner lymphoma OG and RG, Sarcoma 180, Walker 256 carcinosarcoma, Ehrlich ascites, and others (2–6). This discovery led to the hope that the coordination complexes of platinum might represent a new and unique type of antitumor agent and, therefore, might be active against certain human malignancies which are insensitive to presently available cancer chemotherapeutic agents.

Early in the study of *cis*-dichlorodiammine-platinum(II) as an antitumor agent it became evident that it is a rather toxic drug and that it has a low therapeutic index that is very dependent on its purity. Synthesized either by the method of Kaufmann and Cowan (7) from K_2PtCl_4, ammonium hydroxide, and ammonium chloride or by the technique of Dhara (8) via the intermediate K_2PtI_4, *cis*-dichlorodiammine-platinum(II) must be rigorously purified. In our hands, repeated recrystallization from hot dilute hydrochloric acid or by dissolution in

[1] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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dimethylformamide followed by precipitation with dilute hydrochloric acid has achieved this objective most effectively. Several criteria have been employed to assess the efficacy of such purification; they include platinum and nitrogen analyses, infrared spectroscopy, ultraviolet-visible spectroscopy (ratio of A302/A247), thin-layer chromatography, mouse toxicity tests, and others. When prepared and purified in this manner, *cis*-dichlorodiammineplatinum(II) is very effective for the treatment of mouse leukemia L1210. Single doses of 8–10 mg/kg on the third day after tumor transplant produced a 60%–100% increased lifespan (ILS). On the other hand, the narrow therapeutic range for this drug can better be appreciated when one realizes that its LD50 varies from 14 to 20 mg/kg in various strains of mice. Early studies of *cis*-dichlorodiammineplatinum(II) by various investigators (9,10) reported that it was, indeed, a very toxic substance and that it produced significant injury to the bone marrow, the intestinal mucosa, and the distal tubule of the kidney. Such results, taken together with the mouse toxicity data given above, made it evident that this drug was likely to prove a difficult one to utilize in the treatment of human malignancy. Numerous efforts have been made to overcome this obstacle and much that we shall present in the remainder of this report has evolved from such efforts.

In recent years, combination chemotherapy of human acute leukemia as well as other malignancies has clearly demonstrated the superiority of the simultaneous administration of several active antitumor agents as contrasted with the use of a single agent alone. For this reason, the feasibility of combination chemotherapy with *cis*-dichlorodiammineplatinum (II) has been investigated by Venditti et al (11,12) and by investigators at Wadley Institutes (3,13). Table 1 will perhaps illustrate how this agent may be used effectively in combination with other antitumor agents. For example, *cis*-dichlorodiammineplatinum(II) given at a dose of 2.5 mg/kg on Day 3 achieved only a 25%–35% ILS, while cytosine arabinoside given at a dose of 5 mg/kg every 3 hours × 8 on Day 3 produced a 100% ILS when used alone. These two drugs may, however, be combined in optimal doses as indicated to achieve a 200% ILS and 100% survival of all animals treated. Similar synergism was seen when *cis*-dichlorodiammineplatinum(II) was combined with cyclophosphamide to achieve 200% ILS with all animals surviving. This agreed with similar findings by Venditti (12). It is also quite evident that *cis*-dichlorodiammineplatinum(II) and 6-thioguanine may be utilized effectively to achieve synergism in combination chemotherapy. The success of this platinum compound when employed with three other drugs in quadruple chemotherapy is illustrated in table 2. Perhaps the major point to be made is that a great prolongation of life and a high percentage of cures may be achieved with the four-drug combinations including *cis*-dichlorodiammineplatinum(II) though the dose of this drug is appreciably lower than that previously utilized when it is given alone. It would appear that the efficacy of *cis*-dichlorodiammineplatinum (II) can be enhanced or potentiated when it is employed in combination chemotherapy but that toxicities of the individual drugs are not additive. These results would certainly lead one to anticipate that the platinum compound is most likely to prove useful in the treatment of human malignancy in combination with other known antitumor agents.

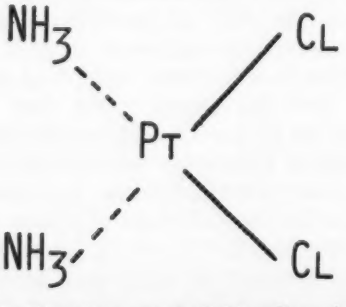

FIGURE 1.—Structure of *cis*-dichlorodiammineplatinum (II).

With certain antitumor agents, therapeutic efficacy has been very dependent on dosage schedule, eg, cytosine arabinoside. *Cis*-dichlorodiammineplatinum(II) has been similarly tested to see if different dosage schedules might improve its therapeutic index. These results are found in table 3. From these data it seems that a single large dose of *cis*-dichlorodiammineplatinum(II) is more efficacious than spaced

TABLE 1.—*Cis*-dichlorodiammineplatinum (II) in optimal combined therapy for murine leukemia L1210*

Drug	Dose (mg/kg)	Day(s) of administration	ILS† (%)	Survivors (%)
Cis-dichlorodiammine-platinum (II)	2.5	3	25–35	0
Cytosine arabinoside	5 (q3h × 8)	3	100	25
Cis-dichlorodiammine-platinum (II) + cytosine arabinoside	As above		200	100
Cis-dichlorodiammine-platinum (II)	2.5	3–6	60	0
Cyclophosphamide	100	3	48	0
Cis-dichlorodiammine-platinum (II) + cyclophosphamide	As above		200	100
Cis-dichlorodiammine-platinum (II)	2	3–6	55	0
6-Thioguanine	10	3	27	0
Cis-dichlorodiammine-platinum (II) + 6-thioguanine	As above		200	100

*Experiments were terminated at 200% ILS.
†Results expressed as % ILS compared with untreated controls.

smaller doses. No improvement in therapeutic index would, therefore, be realized by the latter schedule of treatment. In this connection, it might also be pointed out that these results strongly indicate that this compound is not cell cycle phase-dependent; that is, it seems to act with equal vigor against cells which are actively synthesizing nucleic acids (S phase) or against cells in mitosis (M phase). This finding is somewhat surprising because the work of Howle and Gale (6) indicated that *cis*-dichlorodiammineplatinum(II) given at very low doses selectively inhibited DNA synthesis and therefore one might have anticipated that cells in S phase would be somewhat more sensitive to the drug. On the other hand, Connors (4) has suggested a similarity in the mechanism of action between *cis*-dichlorodiammineplatinum (II) and the bifunctional alkylating agents (eg, melphalan), and indeed, these latter drugs are essentially cell cycle phase-independent also.

In certain instances the toxicity of an antitumor agent has been significantly reduced by concurrent treatment with some other drug (eg, *N*-acetyl-L-cysteine with cyclophosphamide). To a limited extent, we have also explored this possibility in an effort to improve the therapeutic index of *cis*-dichlorodiammineplatinum(II). Table 4 presents the results obtained by concurrent administration of this compound with (1) *N*-acetyl-L-cysteine, (2) spironolactone, (3) salicylamide, or (4) D-penicillamine. Unfortunately, none of these agents achieved the desired result.

Since *cis*-dichlorodiammineplatinum(II) was so promising as a new and unique antitumor agent but yet was so difficult to utilize in human malignancy because of its low therapeutic index, the need to synthesize and test other related platinum complexes was apparent from the beginning. Rosenberg (14) and Cleare and Hoeschele (15) have been particularly active in this approach. The compounds seen in figure 2 were among those which they have reported as promising antitumor agents. These include *cis*-platinum(II)diethanolaminedihalide, *cis*-

TABLE 2.—*Cis*-dichlorodiammineplatinum (II) in multiple-drug combinations

Drug	Dose (mg/kg)	Day of administration	ILS* (%)	Survivors (%)
Cis-dichlorodiammine-platinum (II)	2.5	3	25–35	0
Methotrexate	4			
6-Thioguanine	5	3	66	0
Cyclophosphamide	30			
Cis-dichlorodiammine-platinum (II) + methotrexate, 6-thioguanine, cyclo-phosphamide	As above		200	100
Cis-dichlorodiammine-platinum (II)	2.5	3	25–35	0
5-Fluorouracil	6			
Methotrexate	4	3	60	0
Cyclophosphamide	30			
Cis-dichlorodiammine-platinum (II) + 5-fluorouracil, metho-trexate, cyclophos-phamide	As above		200	100
Cis-dichlorodiammine-platinum (II)	2.5	3	25–35	0
5-Fluorouracil	6			
6-Thioguanine	5	3	50	0
Cyclophosphamide	30			
Cis-dichlorodiammine-platinum (II) + 5-fluorouracil, 6-thioguanine, cyclophos-phamide	As above		200	100

*Results expressed as % ILS compared with untreated controls.

platinum(II)ethylenediamine malonate, *cis*-platinum(II)dichloroethylenediamine, and *cis*-platinum(IV)diamminotetrachloride. In addition to these agents, they and other investigators have synthesized literally scores of other *cis*-dichlorodiammineplatinum(II) analogs, some of which are shown in figure 3. In general, these have proven either too toxic or essentially inactive as antitumor agents. This list is by no means complete and those interested in a more complete listing can obtain this, I understand, from Dr. Rosenberg and his co-workers. We at this institution have also been active in the synthesis and testing of *cis*-dichlorodiammine-platinum(II) analogs. Figure 4 illustrates some of the coordination compounds of platinum prepared and screened in our laboratories. We regret to report that none seems worthy of

TABLE 3.—Time control study*

Day(s) of administration	*Cis*-dichlorodiammineplatinum (II) (mg/kg)	Total dose (mg/kg)	ILS† (%)
3	8	8	100
3	2 (q6h × 4)	8	100
3,4,5,6	2	8	70
3,6	0.5 (q3h × 8)	8	54

*Tumor implant of 10^4 cells ip on Day 0; experiments terminated at 200% ILS.

†Results expressed as % ILS compared with untreated controls.

TABLE 4.—Failure of simultaneous treatment with certain drugs to alter toxicity and/or antitumor activity of *cis*-dichlorodiammineplatinum (II)*

Drug	Dose and schedule	LS ratio† after treatment with indicated drug plus *cis*-dichlorodiammineplatinum (II) at a dose of— 10	20 (mg/kg)	30
Salicylamide	200 mg/kg × 2, Days 3–4	0.80	1.08	0.62
Spironolactone	10 mg/kg, Days 3–7	0.83	1.01	0.82
N-acetyl-L-cysteine	250 mg/kg, Days 3–7	0.92	1.11	0.92
D-Penicillamine	150 mg/kg × 2, Days 3–4	0.99	0.38	1.03

*Treatment of leukemia L1210 in BDF_1 mice.

†LS ratio = $\frac{\text{lifespan with } cis\text{-dichlorodiammineplatinum (II)} + \text{drug}}{\text{lifespan with } cis\text{-dichlorodiammineplatinum (II) only}}$.

further investigation. Our efforts to synthesize coordination compounds of platinum which are active antitumor agents and which have a superior therapeutic index continue and presently we are seeking to synthesize and test the widest possible variety of nitrogen ligands which we can successfully incorporate in the *cis*-dichlorodiammineplatinum(II)-like molecule. Although this program has been remarkably unsuccessful to date, we still have confidence that diligent search will uncover products superior to *cis*-dichlorodiammineplatinum (II).

In 1972, Connors et al (16) described the synthesis and testing of a number of platinum analogs shown in figure 5. The last two compounds in each column seem especially interesting. Their investigation included *cis*-dichlorobiscyclopentylamineplatinum(II) (PAD) and called attention to its efficacy for the treatment of a plasma cell tumor in mice. The structure of this compound is shown in figure 6. Preliminary results from our institute a year later confirmed the work of Connors et al and indicated that PAD was also remarkably effective for the treatment of three other mouse tumors: L1210, MCDV 12, and Gardner lymphoma.[4] Our investigation of this product was recently described.[5] The synthesis is shown in figure 7.

[4] Ridgway H, Lapis S, Hall LM, et al. Platinum compounds and their antitumor effects in mice. Presented at the 2nd International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy, Oxford, 1973.

[5] Speer RJ, Ridgway H, Hall LM, et al. *Cis*-dichlorobiscyclopentylamineplatinum (II) synthesis and animal tests of this new antitumor agent. Presented at the 15th Congress of the International Society of Hematology, Jerusalem, 1974.

PAD was prepared by the interaction of 1 mol of potassium chloroplatinite with 2 mols of cyclopentylamine by agitation of the aqueous solution for 6 hours at room temperature in accord with the directions provided by Connors et al. After the product had been recovered by filtration, it was purified as indicated in figure 8. The importance of this purification procedure warrants emphasis, for the therapeutic index of PAD is very dependent on its purity. Prepared and purified in this manner, it had the theoretical platinum and nitrogen content. As with *cis*-dichlorodiammineplatinum(II), thin-layer chromatography and infrared spectroscopy also proved useful in establishing the identity and purity of the product.

For animal testing, PAD was ground to a very fine dispersion in purified sesame oil and autoclaved in a sealed, evacuated container. It was administered intraperitoneally (ip) to mice and subcutaneously (sc) and intramuscularly (im) to rabbits, cats, and dogs. No serious effects were observed at the injection site. Dispersed in oil in this manner, PAD proved very effective against leukemia L1210, MCDV 12, and Gardner lymphoma OG in mice. These results

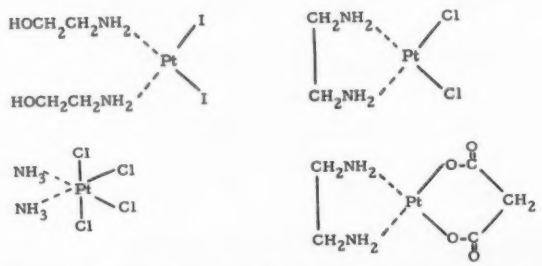

FIGURE 2.—Platinum complexes which have promise as antitumor agents.

trans-$[Pt(NH_3)_2Cl_2]$
cis-$[Pt(NH_3)_2I_2]$
cis-$[Pt(NH_3)_2(NO_2)_2]$
cis-$[Pt(NH_3)_2(SCN)_2]$
cis-$[Pt(NH_3)_2(NO_3)_2]$
cis-$[Pt(MeNH_2)_2Cl_2]$
cis-$[Pt(Me_2NH)_2Cl_2]$
cis-$[Pt(EtNH_2)_2Cl_2]$
cis-$[Pt(Et_2NH)_2Cl_2]$
cis-$[Pt(l\text{-}ProNH_2)_2Cl_2]$
$[Pt(H_2NCH_2CH_2NH_2)C_2O_4]$
$[Pt(MeHNCH_2CH_2NHMe)Cl_2]$
$[Pt(Me_2NCH_2CH_2NMe_2)Cl_2]$
$[Pt(H_2NCH_2CH_2NH_2)_2]Cl_2$
$[Pt(H_2NCH_2CH_2NHCH_2CH_2NH_2)Cl]Cl$
$[Pt(NH_3)_4](PtCl_4)$
$[Pt(o\text{-}Phen)Cl_2]$
cis-$[Pt(Pyr)_2Cl_2]$
$K[Pt(C_2H_4)Cl_3]$
$K[Pt(H_2NCH_2COO)Cl_2]$
$K_2[Pt(C_2O_4)_2]\cdot 2H_2O$
$[Pt(MeNH_2)_2(C_2O_4)]$
cis-$[Pt(MeNH_2)_2Br_2]$
cis-$[Pt(HOCH_2CH_2NH_2)_2I_2]$
cis-$Pt(NH_2OH)_2I_2]$
cis-$[Pt(Et_2S)_2Cl_2]$
cis-$[Pt(P\emptyset_3)_2Cl_2]$
trans-$[Pt(As\emptyset_3)_2(SCN)_2]$
trans-$[Pt(Sb\emptyset_3)_2(SCN)_2]$
$[Pt(Pyr)_4]Cl_2$
$[Pt(EtNHCH_2CH_2NHEt)Cl_2]$
$[Pt(H_2NCH_2CH_2NH_2)(NO_2)_2]$

FIGURE 3.—Platinum complexes which do not seem promising as antitumor agents.

cis-$[Pt(Asp)_2]$
$[Pt(EDDA)Cl_2]$
cis-$[Pt(Asparagine)_2]$
trans-$[Pt(Glutamine)_2]$
cis-$[Pt(H_2NCH_2COO)_2]$
cis-$[Pt(Nicotinate)_2Cl_2]$
cis-$[Pt(Hippurate)_2Cl_2]$
$[Pt(Dimethylglyoxime)_2]$
cis-$[Pt(NH_2CONH_2)_2Cl_2]$
trans-$[Pt(H_2NCH_2COO)_2]$
cis-$[Pt(Cycloserine)_2Cl_2]$
cis-$[Pt(p\text{-}NH_2\emptyset SO_3H)_2Cl_2]$
cis-$[Pt(\emptyset CH_2CH_2NH_2)_2Cl_2]$
cis-$[Pt(p\text{-}NH_2\emptyset COOH)_2Cl_2]$
cis-$[Pt(H_2NNHCONH_2)_2Cl_2]$
cis-$[Pt(4\text{-}Aminouracil)_2Cl_2]$
cis-$[Pt(C_{18}H_{35}NH_2)_2Cl_2]$
cis-$[Pt(n\text{-}C_5H_{11}NH_2)_2Cl_2]$
cis-$[Pt(n\text{-}C_8H_{17}NH_2)_2Cl_2]$
cis-$[Pt(Isonicotinate)_2Cl_2]$
cis-$[Pt(Nicotinamide)_2Cl_2]$
cis-$[Pt(Isonicotinamide)_2Cl_2]$
cis-$[Pt(6\text{-}Mercaptopurine)_2Cl]$
cis-$[Pt(6\text{-}Mercaptopurine)Cl_2]$
$[Pt(HOOCCH_2SCH_2COOH)Cl_2]$
$[Pt(NH_3)_2(OOCCH_2SSCH_2COO)]$
cis-$[Pt(4\text{-}Aminoantipyrine)_2Cl_2]$
cis-$[Pt(HOCH_2CH_2CH_2NH_2)_2Cl_2]$
$[Pt(HOOCCH_2SCH_2SCH_2COOH)Cl_2]$
$[Pt(Bathophenanthrolinedisulfonate)Cl_2]$
cis-$[Pt(Cyclopentylamine)_2(H_2O)_2](NO_3)_2$
cis-$[Pt(HOCH_2CH_2CH_2CH_2CH_2CH_2CH_2NH_2)_2Cl_2]$

FIGURE 4.—Platinum complexes which do not seem promising as antitumor agents (synthesized and tested at Wadley Institutes).

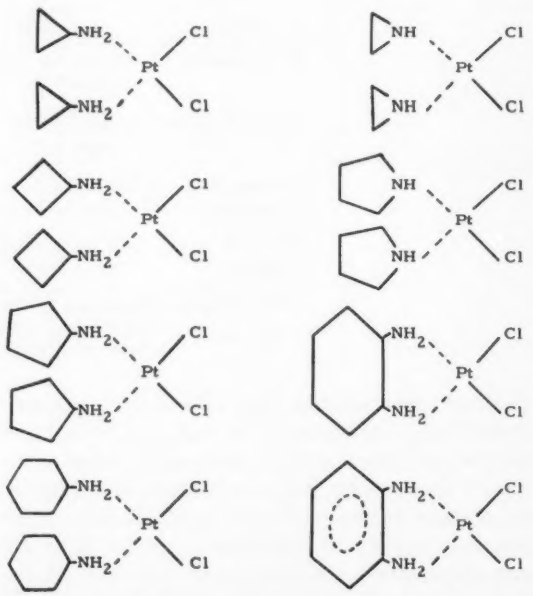

FIGURE 5.—Platinum complexes with antitumor activity.

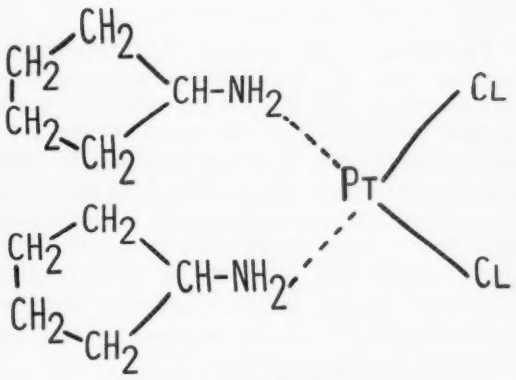

FIGURE 6.—Structure of PAD.

are seen in table 5. Doses of 400–500 mg/kg achieved a significant ILS and a high percentage of cures in all tumors. Little toxicity was seen until doses of 600–800 mg/kg were employed. Rabbits have received up to 300 mg/kg im without obvious toxicity. The sc administration of 100 mg/kg to dogs and cats has been well tolerated.

These findings appeared to warrant initial clinical trials in human malignancy. Accordingly, several terminal cancer patients were treated im with 20–50 mg/kg of sesame oil-dispersed PAD. Subsequent analyses of their urine, blood, and autopsy tissues indicated clearly that the PAD essentially remained at the injection site. In an effort to overcome this obstacle, PAD was formulated as a finely dispersed aqueous sol. To achieve this purpose, PAD was ground to a very fine particle size by prolonged ball milling and then dispersed by wet-ball milling or by sonication in the presence of a suitable suspending agent. Carboxymethyl cellulose (0.2%–1.0%) in physiologic saline was chosen for further investigation; 6% polyvinylpyrrolidone in saline also seems quite promising. PAD formulated in such an aqueous solution cannot be autoclaved in the usual manner but must be pasteurized at 65°C for 1 hour after prior adjustment of the pH to 3.0 with hydrochloric acid. In this formulation, PAD is dispersed as particles which are approximately 1μ–2μ in size, does not settle rapidly, and is compatible with human blood. The product has been given im to rabbits and their tissues were analyzed for platinum. Unfortunately, this product, like that dispersed in sesame oil, essentially remained at the injection site. It does not seem suitable for im treatment of man. PAD as an aqueous colloid has, however, been given orally, sc, ip, and intravenously (iv) to tumor-bearing mice with promising results (table 6). Thus, nearly 100% cure of the MCDV 12 tumor has been achieved with doses of 100–350 mg/kg ip. Doses of 350–500 mg/kg ip have produced a marked prolongation of life in leukemia L1210 but the cure rate for this tumor has been less than that previously mentioned. In general, sc and oral therapy with this drug have been much less effective than ip treatment. The efficacy of iv administration is not fully known at present; 100 mg/kg has achieved an excellent ILS in both tumors but drug toxicity is indicated by the late deaths of all animals. Doses of 50 and 75 mg/kg iv seem to be closer to the optimum for this drug. These results are still rather preliminary and do not necessarily represent the optimal dose for PAD in either tumor and improvement certainly can be anticipated as other doses and dosage schedules are tested.

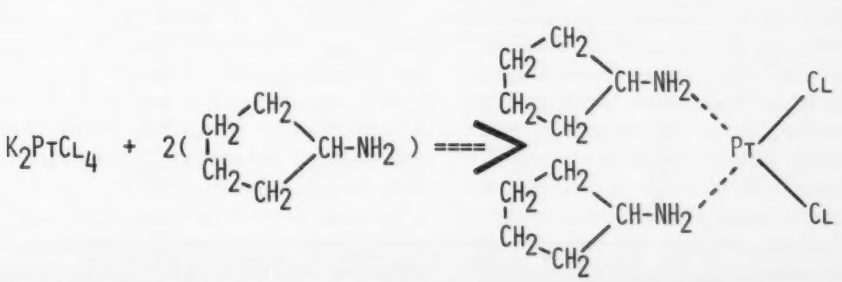

FIGURE 7.—Synthesis of PAD; aqueous solution agitated for 6 hrs at room temperature.

a. Wash with concentrated HCl, water, methanol, acetone, and ether;

b. leach with hot dilute HCl;

c. dissolve in dimethylformamide and adsorb with activated carbon;

d. filter and precipitate with excess dilute HCl;

e. dissolve in dimethylformamide, filter, add equal volume of methanol, chill thoroughly, and filter;

f. wash with water and then methanol and dry at 105° C.

FIGURE 8.—Purification of PAD.

The aqueous sol has also been given iv to rabbits (35–100 mg/kg) and subsequent analysis of their tissues revealed that blood clearance was very rapid and that the liver and spleen accumulated most of the platinum. These results are included in table 7. Sequential chemical and hematologic studies performed on these rabbits suggested only that some slight hepatic cell injury had occurred. Histologic examination of their tissues indicated minimal injury to the liver, lung, and kidney, with more significant damage to the spleen. All other tissues appeared normal. Essentially the same results were obtained when 50 mg/kg was given iv to a healthy dog (*see* table 8).

Preliminary tests on the iv PAD-treated dog showed that his lymphocytes were actively stimulated by phy ohemagglutinin; this would suggest that iv PAD is not an immunosuppressive drug. This is in sharp contrast to most effective antitumor agents and might indicate a unique potential for this product. It would suggest that iv PAD could be combined with immunotherapy (as for example with bacillus Calmette-Guérin) quite effectively. Further experimentation will be required to test the reliability of this prediction.

PAD certainly appears to be a promising and, perhaps, unique antitumor agent. Its insolubility has, however, posed quite a problem to its successful application in man. At this time, it seems that im administration is unlikely to succeed. However, when PAD is properly dispersed as an aqueous colloid, it may yet prove beneficial when employed topically, orally,

TABLE 5.—Antitumor action of PAD in sesame oil*

PAD dose (mg/kg)	Tumor (% ILS)		
	L1210	MCDV 12	Gardner lymphoma OG
500	175	200	184
400	152	200	—
350	151	200	200
300	200	—	—
200	98	200	85

*Single dose ip on Day 3; experiments stopped arbitrarily at 200% ILS.

TABLE 6.—Antitumor effect of aqueous PAD solution*

PAD dose (mg/kg)	Route of administration	Tumor (% ILS)	
		L1210	MCDV 12
500	ip	130	Toxic
350	ip	110	200
100	ip	17	200
350	sc	40	103
500	oral	—	50
100	iv	140†	160†
75	iv	73	—
50	iv	60	—

*Single dose given on Day 3; experiments terminated arbitrarily at 200% ILS.

†Late drug deaths at about 400% ILS.

TABLE 7.—Tissue distribution of platinum after iv administration of PAD to rabbits

Tissues analyzed	Platinum content* (μg/g dry weight) after PAD dose (mg/kg) of—			
	0	25†	35 (Day 0) + 50 (Day 21)†	100‡
Spleen	100	490	950	590
Liver	100	350	260	270
Lung	100	120	140	130
Kidney	120	ND	70	90
Colon	ND	ND	60	100
Small intestine	ND	ND	60	90
Stomach	ND	ND	70	100
Skeletal muscle	110	ND	40	70
Heart	110	90	30	80

*Determined by atomic absorption spectroscopy after ignition and dissolution in aqua regia. ND = not determined.

†Animals killed on Day 36.

‡Animals killed on Day 15.

TABLE 8.—Tissue distribution of platinum after iv administration of PAD to a dog*

Tissues analyzed	Platinum in μg/g dry weight†
Liver	138
Spleen	117
Kidney	106
Gallbladder	74
Colon	53
Lung	42
Small intestine	32
Skeletal muscle	32
Heart	32
Pancreas	32
Stomach	21
Brain	0

*Killed 6 days after 50-mg/kg injection of aqueous PAD solution.

†Determined by atomic absorption spectroscopy after ignition and dissolution in aqua regia.

perhaps ip following paracentesis, by interstitial injection, or most likely by the iv route.

As a result of personal communications from Dr. Rosenberg, in the early spring of 1973, we became actively involved in the synthesis, purification, and animal testing of a new class of platinum coordination complexes termed the "platinum blues." Frankly, we found and still find their synthesis and purification most difficult and perplexing. One mol of *cis*-dichlorodiammineplatinum(II) is dissolved in water and treated with two silver nitrate equivalents to yield the corresponding diaquo derivative. The silver chloride is removed by filtration, and the diaquo derivative, free of chloride ions, is allowed to interact with one mol of uracil or other pyrimidine derivative. This is accomplished by prolonged agitation in the dark at ambient temperature or 37°C for a period of 5–15 days. Generally, dilute alkali is added at the end of the first 24 hours to adjust the pH to 6.5–8.5. Agitation is continued. As the reaction proceeds, the reaction mix becomes a very dark blue although other hues may result when other pyrimidines are employed. Isolation of the resulting platinum blue is generally accomplished by thorough chilling and filtration or by the addition of an excess of ethyl alcohol, chilling, and filtration. The product is usually washed with ethyl alcohol and then with ether and dried in a vacuum desiccator. In our laboratory we have not seen much evidence of storage instability. We have investigated many operating parameters in an effort to improve and standardize this synthetic process; these have included reaction time, temperature, pH, ratio of reactants, concentration of reactants, gas-phase composition, and others, but as yet we feel that we have by no means mastered the process involved. It seems that regardless of what we do, the reaction product is a complex mixture consisting of variable proportions of five or more constituents. It seems evident that

improved synthetic conditions are urgently needed.

We have studied the composition of the reaction product in quite a variety of ways. Some of these are presented in figure 9. We have, for example, subjected the product to electrophoresis on cellulose acetate in a glycine buffer at pH 7.2. Typically, four or five components are seen migrating toward the cathode at different velocities. These are visualized by examination under ultraviolet light, by exposure to iodine vapor and examination under ultraviolet light, and by spraying with stannous chloride for detection of platinum compounds. Other methods of fractionation have included thin-layer and paper chromatography with 0.0001 N nitric acid, paper chromatography with development by dilute hydrochloric acid, thin-layer chromatography on PEI-cellulose using 50% acetone, column gel chromatography using Sephadex dextrans or Bio-Rad polyacrylamide packings with a wide variety of developing solvents, salting-out fractionation, cold alcohol precipitation, selective adsorption, solvent partition, and others. Unfortunately, all have failed to yield a single homogeneous product from the complex reaction product. Despite this failure, we have tested many of these reaction products in leukemia L1210 and MCDV 12 and have satisfied ourselves that they possess some antitumor activity.

During the past 12 months, we have utilized a method of synthesis suggested by Dr. Rosenberg and his colleagues* (fig 10). The close similarity between this process and that already described should be noted. This synthesis has been successfully scaled up in order to begin with 20 g of *cis*-dichlorodiammineplatinum(II); under such circumstances, the yield of fraction A was 8.8 g and that of fraction B, 7.6 g. As far as we can determine, the composition of these fractions has remained essentially the same as the process was scaled up from 1 to 20 g. At present, the product is stored at 2°–4°C in the dark in a vacuum desiccator although the essentiality of these storage conditions is not clearly established. Despite this apparent standardization of synthesis, we must readily admit that both fractions A and B are quite heterogeneous revealing five or more platinum-containing compounds by electrophoresis and/or thin-layer chromatography.

Since this product has been utilized in a variety of animal experiments and in phase I of clinical testing, we would like to comment briefly on some of its properties. It is quite soluble in distilled water (perhaps 100 mg/ml) but appreciably less soluble in physiologic saline. It is virtually insoluble in all organic solvents tested. Though the infrared, ultraviolet, and visible absorption spectra of these products have been studied, the data have not been particularly helpful. Analyses of combined fractions A plus B have yielded results of 48.1%–49.7% platinum content by weight. When this product is to be administered to experimental animals or intended for phase I testing in human malignancy, it is dissolved in sterile distilled water at a concentration of about 75 mg/ml and sterilized by ultrafiltration.

Solutions of platinum blue which I have described have been administered ip, sc, and/or iv to mice, cats, rabbits, and dogs. However, most of our animal testing has used tumor-bearing mice. Table 9 illustrates some of the results obtained. A single dose of 125 mg/kg administered to mice ip on Day 3 after tumor transplant produced a significant ILS in the case of the MCDV 12 tumor but was rather ineffective for the treatment of leukemia L1210. Similar results were obtained when the plati-

a. Electrophoresis on cellulose acetate in a glycine buffer, pH 7.2;

b. paper and thin-layer chromatography developing with 0.0001 N nitric acid, dilute HCl, chelating agents, salts, etc;

c. chromatography on PEI-cellulose with 50% acetone;

d. gel chromatography with Sephadex dextrans and Bio-Rad polyacrylamide columns and many varied developing solvents;

e. selective adsorption (with a variety of adsorbents) and elution;

f. selective solvent extraction;

g. selective alcohol (and other organic solvents) precipitation;

h. liquid-liquid partition;

i. selective salting-out fractionation;

j. others.

FIGURE 9.—Methods tested in an effort to analyze and/or purify platinum uracil blue.

*Rosenberg B, Fisher R, and Peresie H. Personal communication.

a. *Cis*-dichlorodiammineplatinum (II) and (1.0 g, 3.3 mM) and silver nitrate (1.132 g, 6.6 mM) are mixed in 15 ml of distilled water; after 3 hrs, mixing at 25° C and 1 hr storage at 4° C, AgCl is removed by filtration; pH of filtrate is adjusted to 7 with dilute NaOH;

b. 373 mg (3.3 mM) uracil is partially dissolved in distilled water at pH 8.5 by mixing with addition of dilute NaOH;

c. reactants 1 and 2 are mixed at 37° C for 24 hrs; dilute NaOH is added to pH of 6.5; continue reaction 4 more days at 37° C without agitation;

d. chill 6 hrs at 4° C and filter (fraction A); to filtrate add equal volume of ethanol, chill 30 min at 4° C, and filter (fraction B);

e. products stored in desiccator at 4° C;

f. *Yield*: 400 mg fraction A and 350 mg fraction B;

g. *Note*: all operations should be conducted without exposure to light.

FIGURE 10.—Synthesis of platinum uracil blue (*see* footnote 6).

num blue was administered at a dose of 50 mg/kg × 5 on Days 3–7. The product seems relatively nontoxic since doses of 500 mg/kg have produced only occasional deaths in treated mice. Chronic toxicity of this product was determined by weekly ip injections of 125 mg/kg to mice; it was found that the mice continued to thrive and gain weight even after eight such doses. No drug-related deaths were observed. Little evidence of toxicity was noted when the product was given iv to rabbits, cats, or dogs at the doses of 25–100 mg/kg; a slight pyrogenic response was noted in some animals. The repeated application to the eye of the rabbit seemed to cause some irritant or inflammatory reaction.

Histologic examination of tissues from animals treated with platinum blue has shown slight cloudy swelling and rare vacuolar degeneration of the liver suggesting only minimal reversible injury to this organ. The kidney demonstrates focal cloudy swelling and tubular sloughing, both conditions appearing reversible. The spleen (in mice) appeared to be hypercellular as a result of increased numbers of white cells, especially megakaryocytes and polymorphonuclear neutrophils. All other tissues appeared normal.

A limited number of cats and dogs with spontaneous tumors have been treated sc and/or iv with platinum blue. One of three cats with acute leukemia has achieved a remission of about 12 months. A dog diagnosed as having endometrial carcinoma with lung metastases showed a dramatic response with clearing of the lung lesions and reduction of the primary tumor. After 7 months the lungs remained clear and the primary lesion static. Only slight subjective improvement has been seen in other animals treated in the same manner.

The tissue distribution of platinum after the iv administration of platinum blue is indicated in table 10. This healthy dog received 67 mg/kg of platinum blue iv and was killed 6 days later for tissue analysis. You will note that the kidney, liver, spleen, and lung were found to contain the highest platinum content. Information concerning the tissue distribution of platinum in man after iv therapy has been obtained by the analysis of blood, urine, and autopsy tissues during the phase I testing of platinum blue with terminal cancer patients. These data were presented in more detail by Dr. Hill et al (17).

In concluding these remarks, we should like

TABLE 9.—Antitumor activity of platinum uracil blue*

Platinum uracil blue dose (mg/kg)	Day(s) of administration	Tumor (%ILS)		
		MCDV 12	L1210	Gardner lymphoma RG
125	3	160–200	27	
25	3–7	20		
250	3	133	27	
50	3–7	200	16–32	0
500	3	−22 to −60	−58	
100	3–7	175–200	18–26	0

*All mouse tumors were transplanted ip on Day 0; therapy was administered ip with aqueous solutions of platinum uracil blue; experiments were arbitrarily terminated at 200% ILS.

TABLE 10.—Tissue distribution of platinum after iv administration of platinum uracil blue to a dog*

Tissues analyzed	Platinum in μg/g dry weight
Kidney	370
Liver	320
Spleen	180
Lung	170
Small intestine	100
Heart	100
Stomach	90
Testes	90
Gallbladder	70
Injection site	60
Pancreas	30
Skeletal muscle	20
Brain	0

*Dog killed 6 days after iv injection of 67 mg/kg of platinum uracil blue.

to reiterate certain points: (*a*) Dr. Rosenberg and his colleagues have brought to our attention a group of new and unique antitumor agents, the coordination complexes of platinum; (*b*) of the platinum complexes studied to date, *cis*-dichlorodiammineplatinum(II) seems the most promising agent when judged by its efficacy for treating a wide variety of transplantable mouse tumors; (*c*) this drug is rather toxic, has a low therapeutic index, and appears to be cell cycle phase-independent in its action; (*d*) it has already found a limited but very valuable application in human cancer chemotherapy; (*e*) it has shown synergistic potentiation in combination therapy; (*f*) two related platinum complexes, namely, *cis*-dichlorobiscyclopentylamineplatinum(II) and the platinum blues, appear somewhat promising as antitumor agents, and the latter are undergoing phase I clinical trial at this time; and (*g*) many scores of related coordination complexes of platinum have now been synthesized and tested in tumor-bearing animals, but unfortunately they are too toxic or they lack antitumor activity. It still seems possible that other platinum complexes will be discovered that are superior to *cis*-dichlorodiammineplatinum(II) and that they will find useful applications as cancer chemotherapeutic agents. Such work is continuing at our institutes.

LIST OF COMPOUNDS

***N*-acetyl-L-cysteine**: NSC-111180; CAS reg. No. 616-91-1

***Cis*-dichlorobiscyclopentylamineplatinum (II)**: CAS reg. No. 38780-36-8; PAD

***Cis*-dichlorodiammineplatinum (II)**: NSC-119875; CAS reg. No. 15663-27-1; *cis*-platinous diamminodichloride; PDD

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; Cytosar

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-phenylalanine mustard

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-; MTX

D-Penicillamine: NSC-81549; valine, 3-mercapto-, D-

Salicylamide: NSC-3115; CAS reg. No. 65-45-2

Spironolactone: NSC-150399; CAS reg. No. 52-01-7; 17α-pregn-4-ene-21-carboxylic acid, 17-hydroxy-7α-mercapto-3-oxo-, γ-lactone, acetate

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino; TG

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Biologic and Chemical Effects of *Cis*-dichlorodiammineplatinum (II) (NSC-119875) on DNA[1]

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It has been proposed by Harder and Rosenberg (1) that the mechanism for the antitumor activity of *cis*-dichlorodiammineplatinum(II) involves binding of the platinum to cellular DNA. Current knowledge of the mode of binding to DNA has been based largely on model-system studies. From such studies the suggestion has been made that platinum acts like a bifunctional alkylating agent (2) and binds preferentially, but not exclusively, to guanine (3). Recently it has been shown by means of analytic sedimentation (4) that in a competitive reaction between the platinum complex and two different DNAs, the platinum complex prefers to react with the DNA of the higher guanine-cytosine (GC) content. The work presented here confirms guanine as the preferred binding site, proposes a model for the observed stoichiometry, and attempts to relate this binding to observed macromolecular and biologic events.

The absorption spectrum of DNA is markedly altered by reaction of the polymer with *cis*-dichlorodiammineplatinum(II) (5). There is both a large hyperchromic shift and a small bathochromic shift. A similar bathochromic shift is observed for binding of platinum to poly(rA), but very little change can be detected in the absorption spectra of poly(rC) or poly(rG) after binding. Nevertheless, this change in the absorption spectrum of DNA appears to be directly related to platinum binding. The previously accepted method for quantitating the amount of platinum bound to DNA was to measure the change in the ratio of absorbance at 250 nm to that at 270 nm (5). This ratio was determined as a function of r, the mol ratio of platinum complex added to nucleoside in the polymer. From such studies, in which the absorbance ratio decreases from about 1.2 to 0.8, the saturation of the available binding sites has been found to occur at an r value which is dependent upon the GC content of the DNA (table 1). The increasing linear dependence on the GC content of the polymer indicates that there is little or no binding to thymine and that most of the binding occurs in GC-rich regions of the DNA. However, this dependence of saturation r values on the GC content of the DNA also implies that, although reaction of platinum with guanine or cytosine in poly(rC) or poly(rG) has little effect on the absorption properties of the homopolymer, reaction of platinum with guanine and cytosine in DNA has a pronounced effect upon the absorption spectrum.

A less ambiguous method for measuring the amount of platinum bound to the DNA at saturation is by means of ^{195}Pt-labeling of *cis*-dichlorodiammineplatinum(II), which has a 3-day half-life (table 1). As indicated by a comparison of the data from both methods, there is approximately a twofold increase in the saturation r value when radioactive labeling procedures are employed. It is concluded, therefore, that absorbance measurements can detect only about half of the platinum actually bound. This result may reflect the fact that most of the absorbance change observed is due to denaturation, as implied by the hyperchro-

[1] *Cis*-dichlorodiammineplatinum (II): CAS reg. No. 1853-33-9. The ^{195}Pt-*cis*-dichlorodiammineplatinum (II) used in this study was obtained from J. K. Poggenburg of Oak Ridge National Laboratory.

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TABLE 1.—Saturation r values for DNAs and polynucleotides

Polymer	1-Fraction of bases as thymine (*see* refs 6 and 7)	Method of analysis		
		Absorbance titration*	^{195}Pt radioactivity†	Statistical prediction‡
Micrococcus luteus DNA	0.86	0.22	0.34	0.37
Escherichia coli DNA	0.77	0.14	0.26	0.29
Calf thymus DNA	0.72	0.13	0.22	0.25
Hemophilus influenzae DNA	0.70	0.12	0.21	0.24
poly(dT)	0	—	0	0
poly(dA)	1.00	0.46	0.43	0.50

*Absorbance measurements were made in 0.01 M phosphate buffer with a Beckman DK-1A recording spectrophotometer.

†Solutions of a known polymer concentration were allowed to react to completion with excess platinum and then spotted on paper discs. These discs were then washed in either 5% TCA, 0.01 M saline, or water and subjected to scintillation counting.

‡This statistical prediction was calculated by assuming that the probability (P_i) of occurrence for any base (X_i) was equal to the fraction (F_i) of that base in the polymer (6,7); thus, for a base sequence X_iX_j, the probability (P_{ij}) would be F_iF_j. Then, the fraction of the bases bound would be:

$$\sum_{ij} P_{ij} = \sum_{ij} F_i F_j$$

where i, j = adenine, cytosine, or guanine but not thymine. Since one platinum binds to two bases, the corresponding r value is obtained by dividing the fraction of bases bound by 2.

micity, and that denaturation is essentially complete before saturation of the DNA.

Studies with poly(dT) indicate no detectable binding of *cis*-dichlorodiammineplatinum(II); therefore, binding of platinum to the thymine residues in DNA would not be expected. As shown in table 1, the saturation r values, by either method, decrease with increasing thymine content of the polymer. Furthermore, if one assumes that the platinum complex binds to guanine, adenine, and cytosine only in a bidentate intrastrand fashion, a theoretical value of r for saturation of binding sites can be calculated. Such calculated values (table 1) are in good agreement with the values obtained by the ^{195}Pt-labeling technique.

For model systems, guanine derivatives react with *cis*-dichlorodiammineplatinum(II) faster than adenine or cytosine derivatives (8). In order to determine if a similar kinetic perference for platinum binding exists in DNA, ^{14}C-purine-labeled DNA was employed. The complex formed between the platinum compound and the adenine or guanine bases in DNA was stable to mild formic acid hydrolysis, conditions sufficient to completely depurinate the DNA. While purine-platinum complexes remained at the origin, unreacted adenine and guanine moved on the chromatogram with their characteristic R_f values of 0.5 and 0.3 respectively. Consequently, it was possible to quantitate the amount of either adenine or guanine remaining at the origin due to reaction with the drug (table 2). As shown in table 2, about 25% of the guanine was retained at the origin before any significant amount of adenine was affected.

TABLE 2.—Chromatograms of ^{14}C-purine-labeled DNA after mild acid hydrolysis*

	Percent of total ^{14}C-purine counts at R_f of:		
r value	0 (origin)	0.3 (guanine)	0.5 (adenine)
0	0	52	48
0.05	13	40	47
0.10	37	28	36
0.15	49	24	27
Saturation	72	18	10

*Samples were treated in 66% formic acid at 100°C for 15 mins. The hydrolysate was then separated by descending paper chromatography with butanol:water:acetic acid (80:30:12).

Thus, guanine is the preferred site of binding in DNA.

The hyperchromicity change in the absorption spectrum of DNA after reaction with *cis*-dichlorodiammineplatinum(II) amounts to approximately 95% of the total hyperchromicity of the helix and indicates an extensive distortion of the macromolecular structure of the DNA. Since native DNA has a measurable viscosity while denatured DNA has none (9), the denaturation of the DNA by reaction with the platinum complex should reduce the viscosity of the polymer. A rapid loss of viscosity was observed for a 0.1-mg/ml solution of salmon sperm DNA as a function of r. Only 37% of the original viscosity remained at an r value of 0.03.

In order to evaluate the effect of *cis*-dichlorodiammineplatinum(II) on the biologic activity of DNA, the transforming ability of *Hemophilus influenzae* DNA for several drug markers was determined as a function of bound platinum (10). The viability of the total cell population in the absence of antibiotic was unaffected by the addition of the platinum-bound transforming DNA. Thus, any effect on transformation is not due to indiscriminate killing of the whole cell population. Transformation of the host to either streptomycin or novobiocin resistance, assayed by the method of Spencer and Herriott (11), decreased dramatically upon reaction of the transforming DNA with increasing amounts of platinum complex. The mechanism of inactivation was then studied. Uptake of treated DNA was monitored by the method of Randolph and Setlow (12), and integration of treated DNA was measured by the tritium-release method (13). These studies indicate that the uptake of DNA into the cell was unaffected by binding of the platinum complex to the polymer; however, integration of transforming DNA into the host genome was inhibited at a rate which paralleled the loss of transforming ability.

The pharmacologic activity of *cis*-dichlorodiammineplatinum(II) has been attributed to its ability to cause interstrand crosslinks (14). Since crosslinks can presumably inhibit integration of transforming DNA, a comparison was made between the rate of formation of crosslinks and the rate at which the DNA is inactivated. The crosslinking of DNA by platinum can be demonstrated by renaturation phenomena (14). Thus, the rate of formation of platinum crosslinks as a function of r was measured by determining the amount of ^{3}H-thymidine-labeled DNA remaining single-stranded after heat denaturation (10). Two methods for detecting single-stranded DNA were employed: (*a*) equilibrium sedimentation in neutral CsCl, and (*b*) enzymatic digestion with a single-strand specific nuclease from *Aspergillus oryzae*. In both cases, 37% of the DNA remained single-stranded at an r of about 0.02. At this r value, therefore, one interstrand crosslink is formed per molecule of DNA. However, the 37% dose for inactivation of transforming ability corresponds to an r of 0.001. Thus, although one platinum molecule per 1000 bases was a biologically inactivating lesion, the first crosslink occurred when about 20 platinum molecules were bound per 1000 bases. It is concluded, therefore, that interstrand crosslinks are not responsible for most of the biologic inactivation observed. This result is in accord with data obtained on the inactivation of coliphage where one platinum

per phage inactivates the virus while 35 are bound before the first crosslink occurs (15).

In conclusion, a physical model has been proposed for the binding of *cis*-dichlorodiammineplatinum(II) to DNA. According to this model, the platinum complex binds in a bidentate fashion to all the bases in DNA except thymine and exhibits a marked kinetic preference for binding to guanine. At very low doses, this binding results in denaturation of the DNA. Complete denaturation is reached before saturation of all the binding sites. It was proposed that two different types of bidentate bond are formed, eg, bidentate to one strand and bidentate between the strands. The biologic activity of transforming DNA was observed to be very sensitive to bound platinum but the inactivation was not due to interstrand crosslinks.

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Clinical Studies of Platinum Coordination Compounds in the Treatment of Various Malignant Diseases [1,2]

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SUMMARY

Following the prior work of Rosenberg et al, Rosenberg and VanCamp, and Speer et al, we started clinical trials with *cis*-dichlorodiammineplatinum(II) in April 1971. Marked tumor regression was noted in several malignant diseases in these initial clinical studies which were reported at the Chemotherapy Congress in Prague, August 1971. At the present time over 178 patients have been treated with this drug for a variety of malignant conditions. Approximately half of these patients received the platinum compound alone and the other half received it in combination with four other chemotherapeutic agents. Side effects, toxicity, tissue distribution, mode of elimination, dose schedules, and clinical results were analyzed. Depression of platelet levels and toxicity to the kidney tubules limited the dose of the drug. No apparent damage to the kidney was seen in the few patients who received repeated courses of the drug for up to 1 year or more. The good results obtained in patients with squamous cell carcinoma, malignant lymphoma, and endometrial carcinoma seem to indicate that the search for less toxic but equally or more effective analogs should be continued. Initial treatment of patients with *cis*-dichlorobiscyclopentylamineplatinum(II), both in oil suspension and in aqueous suspension, is reported. We also describe the administration of platinum uracil blue at escalating doses in phase I patients.

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Clinical trials of platinum coordination compounds in the treatment of various malignant diseases were started at our institution in 1971. Since that time 209 patients have received platinum compounds. Most of this experience involved the use of *cis*-dichlorodiammineplatinum(II). Recently, clinical studies have also begun with *cis*-dichlorobiscyclopentylamineplatinum(II) (PAD) and platinum uracil blue.

The pioneering work of Rosenberg et al (1–5) was responsible for the development of this growing class of platinum coordination compounds with antitumor activity, and for demonstrating the effectiveness of *cis*-dichlorodiammineplatinum(II) in tumor-bearing animals. The usefulness of this drug in the treatment of

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[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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animal malignant diseases was further extended by the observations of Talley (6), Venditti (7), and Welsch (8–10).

At the Wadley Institutes, *cis*-dichlorodiammineplatinum(II) was first employed in phase I clinical trials (11) after the extensive tests with this drug in L1210 leukemic mice by Speer et al (12). Toxicity studies in rats, rabbits, and dogs were also performed. In our initial use of the platinum compound in two patients in the terminal phases of malignant disease, a significant reduction of tumor mass ($> 50\%$) was observed as was a modest prolongation beyond the expected survival. Severe degeneration and necrosis of kidney tubules found at autopsy clearly indicated that the single dose given exceeded tolerable levels for subsequent clinical use. These studies demonstrated that the drug does produce tumor regression, the dose is limited by nephrotoxicity, and side effects include nausea and vomiting (but less severe gastrointestinal damage than in animals). In addition, we obtained information on the following: (*a*) rate of uptake from the blood stream, (*b*) tissue distribution, and (*c*) rate of elimination through the urine. Toxic effects not anticipated from animal studies were tinnitus, deafness, and central nervous system toxicity indicated by disorientation, delerium, and coma.

The safe single dose was set at 2.5 mg/kg of body weight given intravenously (iv) at a rate not to exceed 1 mg/kg/hour (13). Additional experience indicated a total course dose limit of 4–6 mg/kg over a period of 1–2 weeks. At these doses rapid regression was observed in two patients with lymphosarcoma, one patient with advanced Hodgkin's disease, and one patient with acute lymphatic leukemia, and clinical improvement with 1-year survival was noted in a patient with bone metastases from carcinoma of the kidney. Surprisingly, this latter patient received 8 mg/kg of *cis*-dichlorodiammineplatinum(II) over a 10-day period with only moderate temporary decrease of kidney function in the remaining kidney.

Since our first report, clinical studies have continued with platinum therapy alone and also with various combinations with cyclophosphamide, methotrexate, 6-thioguanine, vincristine, bleomycin, 5-fluorouracil (5-FU), and vinblastine.

By the time of the Second International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy at Oxford in 1973, a concensus was emerging concerning susceptible tumors in man, doses, schedules of treatment, toxicity, and side effects. Excellent results were reported in squamous cell carcinoma of the skin, larynx, and esophagus, as well as in endometrial carcinoma, acute myelogenous leukemia (13), ovarian carcinoma (14), and testicular tumors.

More recently, Higby et al (15) noted very good results with the platinum compound in nine of 11 patients with testicular tumors and also observed good responses in carcinoma of the bladder, breast, and thyroid. Talley et al (16) observed objective remission in neuroblastoma, malignant thymoma, and three of five lymphomas. Lippman et al (17) observed "significant toxic side effects and modest therapeutic benefits" mostly in squamous cell cancers.

Kovach et al (18) found *cis*-dichlorodiammineplatinum(II) to be inferior to 5-FU for colon carcinoma. However, Ellerby et al (19) using 5-FU combined with this agent obtained over 50% tumor regression in nine of 25 patients. A 50% tumor regression was noted in four of nine patients with colon carcinoma, one of whom had previously failed to respond to 5-FU including treatment by intrahepatic arterial infusion. Of these patients with $\geq$ 50% responses, mammary, jejunal, and tonsillary adenocarcinoma were included.

Rosenberg (20) has compiled the results of platinum therapy reported by many clinicians for phase I trials. The 28 tumors listed as responding by objective criteria indicate the broad spectrum of antitumor activity. Even if we consolidate some of the tumors according to tissue or organ of origin, we still have 18 varieties, an impressive number of diverse neoplasms.

Dose, rate of iv administration, therapy schedule, and interval between courses have varied considerably with different investigators. With a few exceptions recommended single-dose levels have varied from less than 0.5 to 2.5

mg/kg. The total dose of *cis*-dichlorodiammineplatinum(II) per course varied from 1.25 to 8 mg/kg (11,17) over 10 days. Speed of administration may be of considerable importance but there are very little animal or clinical data on this point. Toxicity studies in beagles[5] showed moderately long survival with slow administration versus iv push. Only one animal received as small a dose as 4 mg/kg in this experiment and this animal survived. Clinical observations seem consistent with this concept (13).

There seems to be no clear evidence in patients that fractionating the dose is superior to giving two or three doses at 24–48-hour intervals. If course doses exceeding 2.5 mg/kg are to be given, it is necessary to give multiple administrations suitably spaced.

Combination chemotherapy with platinum is well recommended on the basis of numerous animal studies. However, clinical reports are very scanty on this point. In our earlier reported therapy, combining three or four agents with platinum gave disappointing results when all types of tumors were considered together.

A great variety of other platinum compounds have been synthesized, starting with the work of Rosenberg (20) and Cleare and Hoeschele (21). One compound which has interested us was first synthesized by Connors et al (22) in 1972. This was *cis*-dichlorobiscyclopentylamineplatinum(II) (PAD), which they demonstrated to be effective in the treatment of a plasma cell tumor in mice. This was confirmed by Speer et al (23), who also found this preparation effective in the treatment of mouse tumors such as L1210 leukemia, MCDV12 leukemia, and Gardner lymphoma (23). A dispersion of finely ground PAD in purified sesame oil as used in animals was ineffective in patients because of the failure to leave the site. Speer et al (24) have prepared PAD as an aqueous colloid in polyvinylpyrrolidone and made it available to our clinical service. Another very interesting platinum compound, platinum uracil blue, prepared by Speer et al (23) according to a personal communication with Dr. Rosenberg, was made available for our clinical use after his experience in animals.

[5] Hill JM, et al. Unpublished data.

MATERIALS AND METHODS

Cis-dichlorodiammineplatinum(II) was prepared in our chemistry laboratory as previously described by Speer et al (23). Just prior to use the dried material was dissolved in 1 mg/ml of sterile normal saline and the pH was adjusted with hydrochloric acid to 2.9. As an extra precaution the materials were run through a millipore filter before administration. The preparation was then given iv without further dilution using a volutrol to hold the rate at 1 mg/kg/hour. The larger person would thus receive the material faster than a child or smaller person. With the exception of some of our very early work, this material has been given as a single injection in the range of ½–2½ mg/kg. Schedules have varied from ½ mg twice daily to the maximum single dose of 2½ mg. Repeated dose schedules have been used over a period of 5–10 days with intervals of 1 or more days. A frequently used schedule was an initial loading dose of 2 mg/kg with 1 or ½ mg/kg given 2 or 3 days later. Total course doses varied ordinarily from 2 to 4 mg with an interval between courses of at least 1 month. Treatment was pursued until limiting toxicity or complete remission was observed.

To lessen the common side effects of nausea and vomiting, the patient was prepared an hour before therapy with antiemetics. *Cis*-dichlorodiammineplatinum(II) was usually given late in the afternoon and the patient frequently would sleep through the night with anorexia rather than experiencing nausea or vomiting the next day. The following laboratory tests were performed before and after treatment: complete blood count (including platelets), creatinine clearance, blood urea nitrogen (BUN), uric acid and cholesterol, alkaline phosphatase, blood sugar, total protein and serum glutamic oxaloacetic transaminase (SGOT), and lactic dehydrogenase. A creatinine clearance level of less than 50 ml/minute was, with rare exceptions, considered a contraindication.

Several different combinations with *cis*-dichlorodiammineplatinum(II) were given, with variations according to the tumor treated. One of these combinations involved a single dose of the platinum compound at 2 mg/kg, metho-

trexate, cyclophosphamide, and 6-thioguanine. Other combinations consisted of platinum followed by 5-FU, cyclophosphamide, vincristine, and methotrexate, and platinum combined with bleomycin and either vincristine or vinblastine. PAD used clinically was prepared by Speer et al as they described in their studies in animals (24), and was administered intramuscularly (im) into the gluteal region at doses of 20–50 mg/kg. More recently, the PAD aqueous colloid suspended in polyvinylpyrrolidone has been given iv over a 5–10-minute period. The same preparation was used intraperitoneally (ip) at doses of 15 and 30 mg/kg.

Platinum uracil blue was prepared by Speer et al (23) and was stored in a vacuum dessicator as a dry powder. It was dissolved in sterile water and sufficient saline was added to bring the preparation close to isotonicity. Initial dosages for patients were started at 30 mg/kg and increased to 100 mg/kg.

RESULTS

As previously reported, *cis*-dichlorodiammineplatinum(II) was removed rapidly from the circulation and was widely distributed in the tissues with less than 10% of the platinum remaining in the plasma 1 hour after administration. After this time, the half-life in the blood stream varied somewhat from 2 to 5 days. Platinum was eliminated chiefly by the kidney with the peak of excretion in the first 48 hours.

Tissue distribution following drug administration is shown in table 1. These tissues were removed at autopsy. The patient with Hodgkin's disease died 2 days after drug administration, and the patient with lymphosarcoma died 25 days after administration. Both patients received a single dose of 2 mg/kg. Note the widespread distribution with a tendency for the greatest concentration to be in the kidney shortly after administration and later in the liver. Figure 1 shows the urinary excretion of platinum uracil blue in four patients. Table 2 shows the tissue analyses of patients receiving platinum uracil blue as compared to a patient with acute granulocytic leukemia who had never received platinum preparations. An interesting feature of this distribution study is a very high level of platinum in the thyroid of a 4-year-old child with acute leukemia. Again, we find high levels in the liver and kidney, but also in the spleen and, in one patient at least, the tumor.

Data on PAD uptake, tissue distribution, and excretion are not yet available, but because of the particulate nature of the preparation we would expect it to have a reticuloendothelial distribution.

Cis-Dichlorodiammineplatinum(II) Therapy

Table 3 shows the toxic effects observed clinically in 78 patients with various malignant diseases other than leukemia treated with *cis*-dichlorodiammineplatinum(II). These represent those effects of sufficient degree to be brought to the attention of the nurse or physician and may not include some very mild degrees of discomfort. The table also might exclude those effects experienced during the night or not reported by the patient. Nausea and vomiting was by far the most common side effect, with diarrhea, tinnitus, and deafness all being observed in 3% or less of the patients. Mild degrees of hearing loss were probably also not detected since audiometer tests were not performed. A variety of symptoms seen with other chemotherapeutic agents were not observed, such as alopecia, mucous membrane lesions, epigastric distress or pain, urticaria, pruritis, or peripheral neuritis.

Laboratory evidence of toxicity is shown in figures 2–5 as taken directly from the computer. Each ordinate represents an average of those determinations taken during approximately 60 days after the administration of the platinum. The symbols used indicate the number of pieces of data in the ordinate. For example, Y represents the average of determinations for over 50 patients, L represents 26–50 patients, T represents 11–25 patients, X represents six to ten patients, V represents two to five patients, and I represents one patient. Figure 2 shows the effect of the platinum compound on the leukocyte count in a variety of tumors (excluding leukemia) involving 78 patients. It is evident that platinum has a moderate depressing effect on the leukocyte count, the nadir of depression occurring on about the 28th day. Note that only 9% of the patients had a count at

any time below 1000 cells/mm³, 22% below 2000 cells, and 49% below 4000 cells.

Figure 3 shows a similar analysis of the effect of platinum therapy on the platelet count. The nadir of depression is at the 17th or 18th day, but the average at the most depressed point is still a very comfortable level of 240,000 platelets/mm³. There were, however, a few patients who exhibited severe depression (eg, a minimum platelet count of 0 cells/mm³ was found for a 3-day period in one patient receiving a dose of 5½ mg/kg).

Figure 4 shows the averages of BUN determinations after platinum therapy. There was a fairly sharp rise, with the maximum occurring about the ninth day after therapy. Note that 17% of the patients had values greater than 40 mg/100 ml, 28% greater than 30 mg/100 ml, and 55% greater than the normal upper limit of 20 mg/100 ml. This leaves approximately 50% of the 78 patients who did not show BUN determinations higher than normal.

Figure 5 shows the effect of *cis*-dichlorodiammineplatinum(II) on the SGOT level. Surprisingly, despite the heavy concentration of platinum in the liver, the SGOT showed a rather moderate elevation (when the upper limit of normal is determined to be around 40 milliunits/ml and when values up to 90 milliunits/ml are determined to be of minor significance). The nadir of the effect occurred at about the eighth or ninth day after treatment.

Complete regression of a number of tumors was observed during the period of study. Complete regression was defined as no detectable

TABLE 1.—Tissue analysis after treatment with *cis*-dichlorodiammineplatinum(II)

Organ	μg of drug/g of dry tissue in patient with— Hodgkin's disease* (2 days after therapy)	Lymphosarcoma* (25 days after therapy)
Liver	9	24
Colon	4	3
Stomach	7	5
Small intestine	4	4
Kidney	15	6
Testes	9	9
Pancreas	4	6
Lymph node	9	<3.6
Spleen	6	6
Adrenal gland	4	<3
Lung	7	8
Thyroid	6	5
Muscle	5	3
Brain	2	<2
Gallbladder	11	7
Heart	4	3
Thymus	9	—

*Drug dose: 2 mg/kg.

TABLE 2.—Tissue analysis after treatment with platinum uracil blue*

Organ	μg of drug/g of dry tissue in patients with— Acute granulocytic leukemia (no drug given)	Acute granulocytic leukemia (20 mg/kg)	Carcinoma of the tongue (60 mg/kg)	Carcinoma of the lung (70–100 mg/kg)
Liver	83	344	598	1193†
Spleen	24	86	587	1568
Kidney	83	91	424	943
Tumor	—	22	206	1136†
Thyroid	60	2096	—	80
Lung	95	43	348	261
Pancreas	48	54	43	144
Gallbladder	—	47	120	216
Lymph node	60	—	43	239
Bone marrow	—	54	22	148

*There was no significant difference from tissues of patient receiving no platinum in the adrenal gland, brain, colon, fat, heart, large intestine, ovary, skeletal muscle, skin, small intestine, spinal cord, stomach, and testes.

†Large liver and spleen.

TABLE 3.—Toxic effects from *cis*-dichlorodiammineplatinum(II) in 78 nonleukemic patients

Toxic effects	No. of patients	% Occurrence
Anorexia	3	3
Nausea	31	39
Vomiting	24	30
Severe nausea	3	3
Severe vomiting	4	5
Diarrhea	3	3
Ringing in ears	3	3
Deafness	2	2

tumor, but without a designated time limit before reappearance. Using this definition, complete regression was observed in 13 patients. Of this group, those receiving the platinum compound alone included three patients with squamous cell carcinoma of the head and neck, one with seminoma, one with lymphosarcoma of the stomach, two with endometrial carcinoma, and one with myelogenous leukemia. The latter response was the only complete remission among 40 patients in various stages of acute leukemia who received the platinum drug. In the combination therapy group, responses were noted in one patient with squamous cell carcinoma of the head and neck, one with bronchogenic carcinoma of the lung (squamous cell type), one with carcinoma of the pancreas, one with rhabdomyosarcoma, and one with carcinoma of the breast with lung metastasis.

Table 4 shows a breakdown of all of the patients in the nonleukemic group according to their results of therapy with *cis*-dichlorodiammineplatinum(II) alone. The 71 patients receiving the drug in combination are listed in table 5 according to tumor category and degree of improvement or regression of the neoplasm. The results in this total combination therapy group were somewhat poorer than those in the group receiving *cis*-dichlorodiammineplatinum(II) alone, with the exception of squamous cell carcinoma. In this instance, the 40% response rate (> 50% regression) is definitely superior.

Because of the somewhat cumulative toxic effects of *cis*-dichlorodiammineplatinum(II) on the kidney, it seems worthwhile to note that in our 78 nonleukemic patients treated with

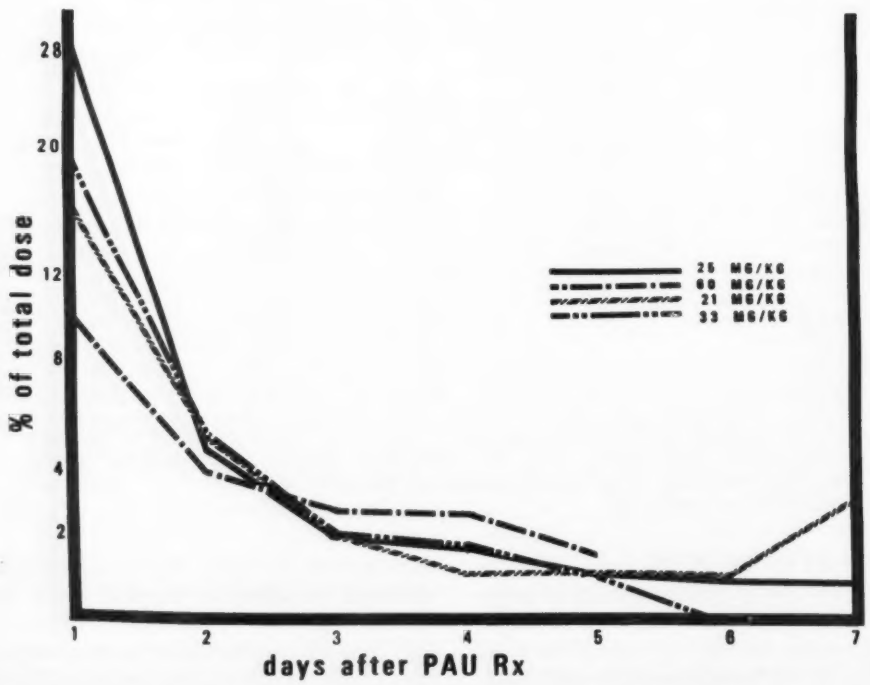

FIGURE 1.—Urinary excretion of platinum uracil blue (PAU).

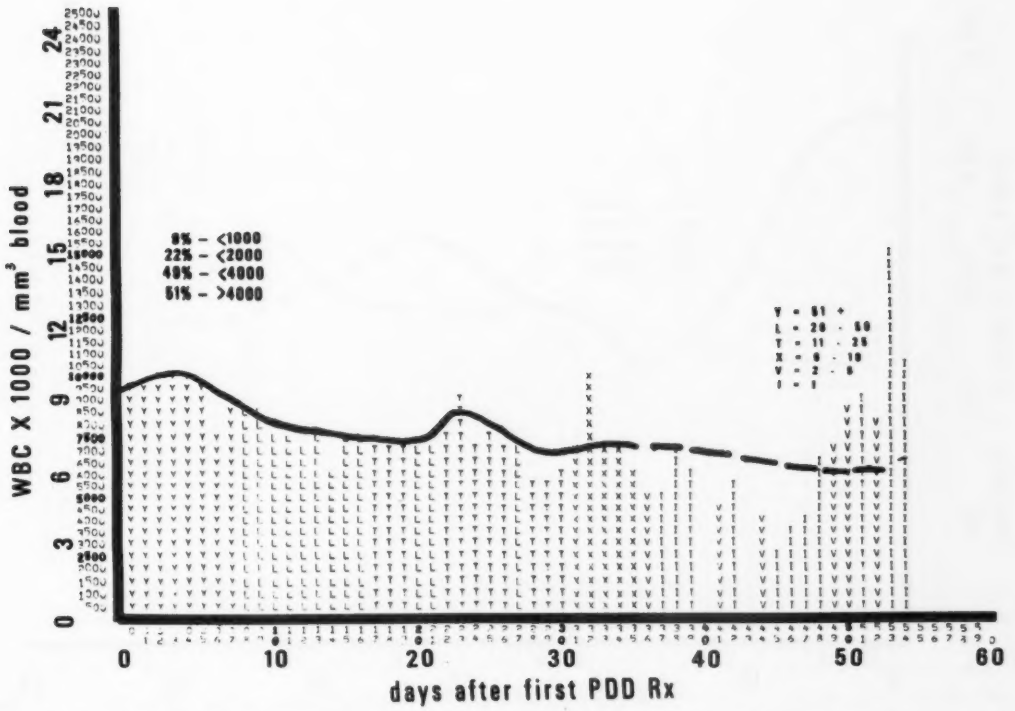

FIGURE 2.—Effect of *cis*-dichlorodiammineplatinum(II) (PDD) on white blood cell counts. Averages of 78 patients excluding those with leukemia. Y = average of determinations for >50 patients, L = 26–50 patients, T = 11–25 patients; X = 6–10 patients; V = 2–5 patients; I = 1 patient.

this drug alone (table 6), one patient had ten courses of therapy, one had eight, one had seven, three had five, six had four, 16 had three, 23 had two, and 27 had one. It must be noted that therapy with the platinum drug was interrupted if the creatinine clearance dropped below 50 ml/minute and was not used again until the creatinine clearance levels again rose above that figure. With this regimen no permanent kidney damage sufficiently severe to contribute to the patient's death was observed.

Case Reports

Patient No. 1.—Patient No. 1 was a 66-year-old white woman who died of squamous cell carcinoma of the right tonsil and right larynx, representing two primaries. This case is a good example of *cis*-dichlorodiammineplatinum(II) therapy in a responsive tumor type. Previous treatment consisted of radiotherapy to both areas with recurrence 1 year later. Her initial treatment with the drug was 3 mg/kg in two doses separated by a 1-day interval. Two additional courses of 2 mg/kg each at monthly intervals totaled 7 mg. After these three courses of therapy the patient improved until there was no definite evidence of disease noted by the ear, nose, and throat surgeon. A month later, however, recurrence was noted and she was changed to bleomycin therapy for 1 month because of diminution of the creatinine clearance to 48 ml/minute. The creatinine clearance rose again to 60 ml/minute and the drug was given in ½-mg doses twice daily followed immediately by cyclophosphamide, 5-FU, vincristine, and methotrexate. For the next 7 months she received no additional *cis*-dichlorodiammineplatinum(II) but had a course of adriamycin and bleomycin at about 2-month intervals. However, there was some progression of the disease, and

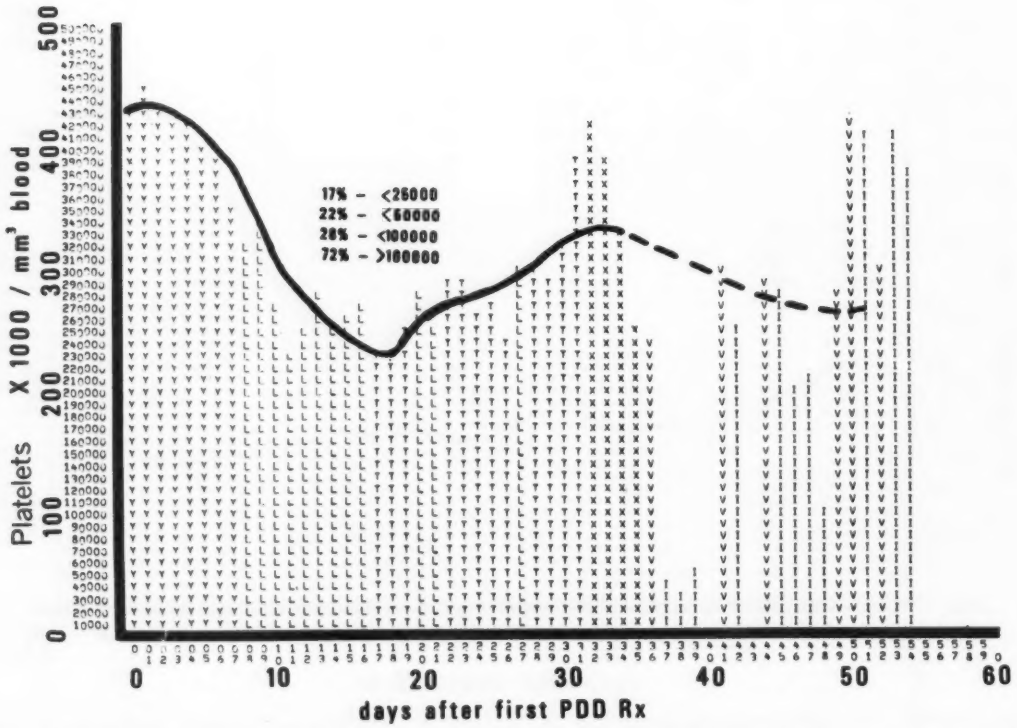

FIGURE 3.—Effect of *cis*-dichlorodiammineplatinum(II) (PDD) on platelet counts. Averages of 78 patients excluding those with leukemia. *See* legend to fig 2 for explanation of codes.

TABLE 4.—Results of therapy with *cis*-dichlorodiammineplatinum(II) in 78 nonleukemic patients

Neoplasm	No. of patients	% of patients with improvement-regression		
		<25%	25%–50%	>50%
Genitourinary	3	0	33	67
Lymphoma	3	33	0	67
Hepatoma	2	50	0	50
Sarcoma*	8	62	0	38
Squamous cell	31	58	19	23
Lung	7	72	14	14
Gastrointestinal	14	86	7	7
Metastatic adenocarcinoma	1	0	100	0
Astrocytoma	1	0	100	0
Breast	6	67	33	0
Myeloma	2	100	0	0
Total	78	61	17	22

*Reticulum cell sarcoma (5 patients), rhabdomyosarcoma (1 patient), osteogenic sarcoma (1 patient), leiomyosarcoma (1 patient).

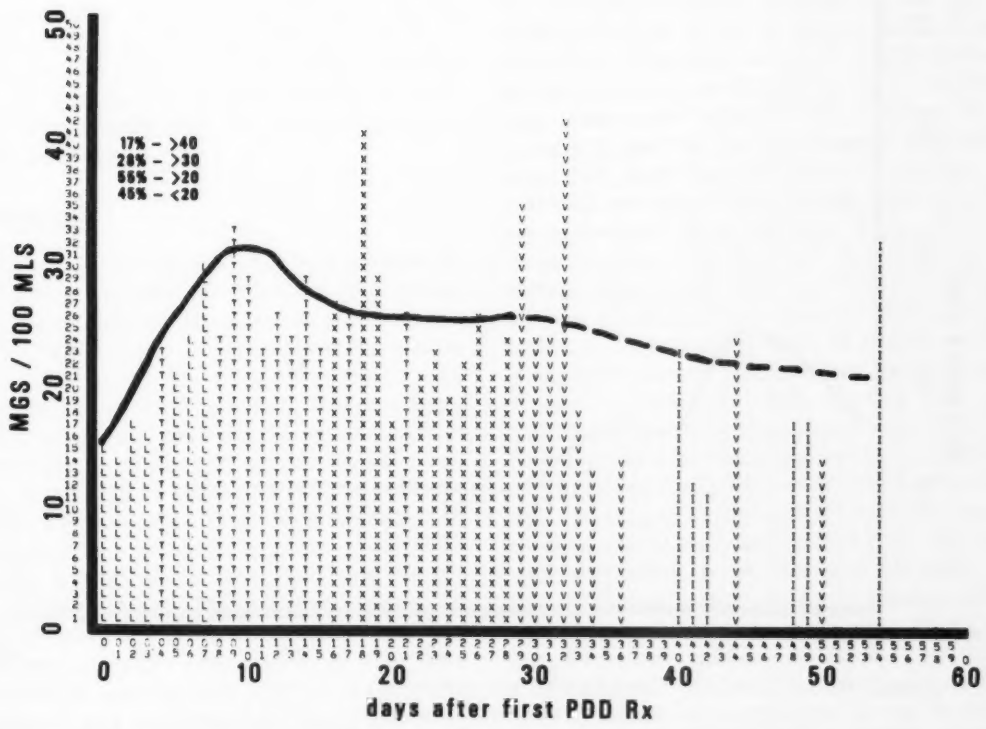

FIGURE 4.—Effect of *cis*-dichlorodiammineplatinum(II) (PDD) on BUN levels. Averages of 78 patients excluding those with leukemia. *See* legend to fig 2 for explanation of codes.

TABLE 5.—Results of combination therapy with *cis*-dichlorodiammineplatinum(II) in 71 nonleukemic patients

Neoplasm	No. of patients	% of patients with improvement-regression		
		<25%	25%–50%	>50%
Pancreas	3	67	0	33
Squamous cell	10	50	10	40
Sarcoma*	5	40	40	20
Genitourinary	11	64	27	9
Breast	11	81	9	9
Gastrointestinal	7	57	43	0
Lymphoma	5	60	40	0
Hodgkin's disease	4	75	25	0
Lung	9	89	11	0
Carcinoma, miscellaneous	5	100	0	0
Myeloma (multiple)	1	100	0	0
Total	71	69	20	11

*Rhabdomyosarcoma (2 patients), synovial cell sarcoma (1 patient).

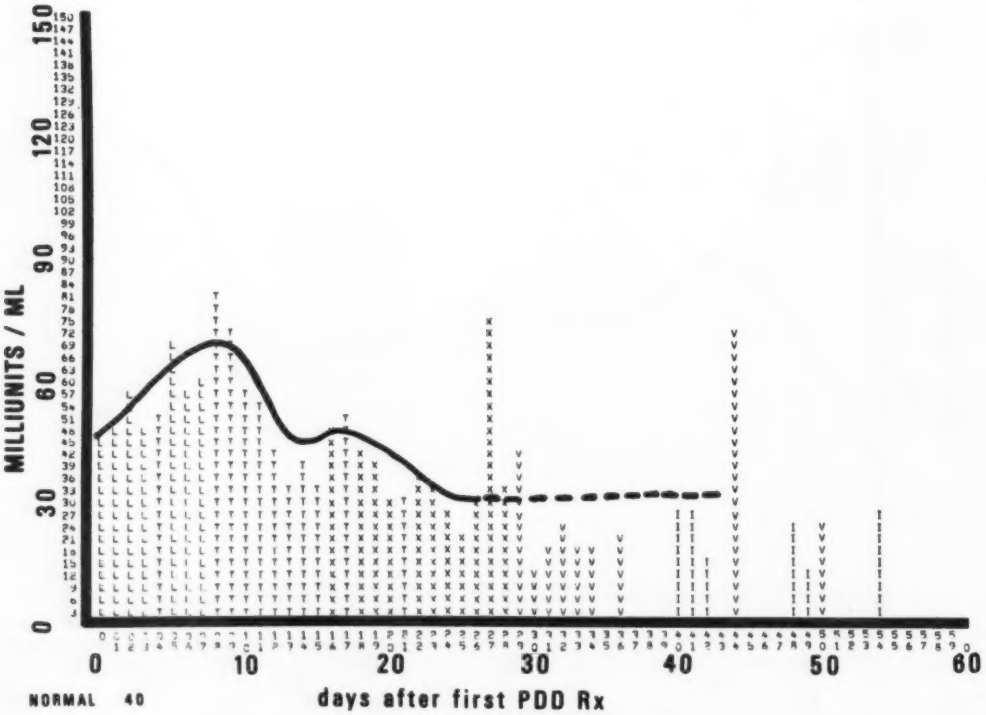

FIGURE 5.—Effect of *cis*-dichlorodiammineplatinum(II) (PDD) on SGOT levels. Averages of 78 patients excluding those with leukemia. *See* legend to fig 2 for explanation of codes.

TABLE 6.—Courses of therapy in 78 nonleukemic patients

No. of courses	No. of patients
1	27
2	23
3	16
4	6
5	3
6	0
7	1
8	1
9	0
10	1

two doses of platinum uracil blue (60 and 80 mg/kg) were administered on two occasions separated by about 2 weeks. Neither significant side effects nor results were noted. At the end of October this patient again received 2 mg/kg of *cis*-dichlorodiammineplatinum(II) iv with a creatinine clearance at the time of administration of 57 ml/minute. This patient is presently still alive but with some tumor present which is being treated with additional courses. This represents survival of 1 year beyond the prognosis made by the referring surgeon.

Patient No. 2.—Patient No. 2 is a 73-year-old white woman who had squamous cell carcinoma of the left cheek. She had previously received extensive radiation therapy and four courses of quadruple chemotherapy. The lesion appeared to regress completely after this treatment but it returned during therapy 5 months later. During the next year she received nine courses of *cis*-dichlorodiammineplatinum(II) at 2 mg/kg in combination with methotrexate, cyclophosphamide, and 6-thioguanine. Between the second and third course of therapy the tumor completely disappeared and no evidence of recurrence has been observed up to the present time, 3 years after the start of the platinum combination chemotherapy. During the course of the platinum treatment, the BUN and cre-

atinine levels in the serum were within normal limits. Now, at the age of 76, this patient is having a gradual rise in these determinations up to 36 mg/100 ml for the BUN and 2.3 mg/100 ml for the creatinine clearance. Unfortunately, at no time has it been feasible to do a creatinine clearance test due to circumstances beyond our control.

PAD Therapy

Eight patients received PAD in a sesame oil slurry given by deep im injection. No tumor regression or side effects were observed except local irritation and, in two patients, sterile abscess. Following the successful use of an aqueous colloid of PAD in polyvinylpyrrolidone, three patients have received this material. The first patient, a child with terminal acute leukemia, was given 10 mg/kg iv with no adverse reaction. The second patient was a woman with carcinoma of the breast in terminal phase with extensive metastases in the liver and in the fluid of the abdomen. This patient received 30 mg/kg given ip with no short-term side effects. The third patient, a young woman with liposarcoma with primary site between the bladder and rectum, had numerous peritoneal implants. This patient received 15 mg/kg ip and 10 mg/kg iv. Again, there were no short-term side effects. Too little time has lapsed to assess any therapeutic results.

Platinum Uracil Blue Therapy

Twelve patients have received platinum uracil blue iv with no evidence of any type of toxic reaction or side effect. Doses began at 20 mg/kg and were slowly escalated to 100 mg/kg. No significant therapeutic results have been obtained at these levels. However, the 13th patient treated warns of a possible very adverse effect from this agent. The patient, a 65-year-old white man, was diagnosed with carcinoma of the lung with pleural effusion. Previous therapy consisted of cyclophosphamide and mechlorethamine, the latter being given into the pleural cavity. A bone scan indicated metastases to the posterior portion of the first left rib. There was also an abnormal liver scan with hepatomegaly and uneven uptake in the right lower and left lobe. Pulmonary function studies showed severe obstructive and restrictive pulmonary defect. Roentgenograms showed nodular density 2 cm in size in the left midlung field. Platinum uracil blue administered by the iv route was started about 10 AM, but it was interrupted due to the reaction to be described, and the patient actually received an estimated total dose of only 44 mg/kg. The material was given very slowly, the total estimated volume being 36 ml. After about 45 minutes from the start of the therapy, the patient complained of a sensation of burning all over his body. When questioned he admitted to being only slightly short of breath with no chest pain. He was pale, restless, and sitting up in bed. Immediately upon hearing this complaint, the platinum blue was discontinued and benadryl and prednisolone were immediately administered iv. Ten minutes later prednisolone was given again (500 mg). It was not possible to obtain a blood pressure reading; the pulse was somewhat weak but not thready. The patient's respiration suddenly ceased and he collapsed, no heart sounds, pulse, or respiratory effort being noted. Artificial respiration (AMBU-BAG) and external cardiac massage as well as intracardiac epinephrine and iv sodium bicarbonate were administered. Aramine drip was also started. A fairly good pulse was observed after these measures, but no voluntary respiratory efforts were made up to the time of death. An autopsy was requested but denied. The day before the therapy the arterial PO_2 was 56 mm Hg compared with a minimum PO_2 at this age of 85 mm Hg. On the day of therapy blood hemoglobin was 9.6 g/100 ml and a blood transfusion had been ordered but not given. Oxygen had also been given by mask when severe distress appeared. The patient was fever-free on the day of therapy. Prior to the administration of platinum uracil blue to this patient, the same preparation had been given to other patients without adverse effects and with no evidence of side effects other than slight temporary discomfort over the vein during administration.

DISCUSSION

The platinum coordination compounds con-

tinue to hold promise in cancer chemotherapy. *Cis*-dichlorodiammineplatinum(II) has been sufficiently tried in clinical use to establish a definite place in the therapy for squamous cell carcinoma, testicular, ovarian, and endometrial tumors, and lymphosarcoma. A wide spectrum of malignant diseases have also responded but additional data are required including results of combination therapy to establish the value of this platinum compound in this more comprehensive role. Combinations with bleomycin, vincristine, 5-FU, cyclophosphamide, cytosine arabinoside, and methotrexate are promising for selected tumors.

The major limiting toxicity is damage to the kidney tubules. This effect ordinarily is dose related but may, in rare instances, be severe after minimal doses of *cis*-dichlorodiammineplatinum(II). Prior kidney disease or concomitant use of nephrotoxic drugs or antibiotics may seriously impair kidney function.

It is essential to monitor kidney function before and during therapy. A creatinine clearance of less than 50 ml/minute is probably a contraindication for *cis*-dichlorodiammineplatinum(II) therapy. Nevertheless, significant reversal of poor kidney functioning is possible and multiple courses of platinum therapy up to a total of ten courses have been possible in this series of patients.

While *cis*-dichlorodiammineplatinum(II) may depress platelet production and to a lesser extent granulocytes and red blood cells, this has not been a problem in our series at recommended doses up to 2.5 mg/kg. We have not observed permanent marrow damage, and furthermore, platelet concentrates, red blood cells, and granulocyte concentrates are available to carry the patient through a temporary marrow depression.

For PAD, phase I clinical studies are still necessary to determine dosage, therapeutic value, and possible toxicity and complications. PAD in aqueous colloid apparently can be administered iv or ip. Because of its successful ip use in animals, this type of administration may offer some advantages especially with peritoneal implants from abdominal tumors.

Clinical studies of iv platinum uracil blue also seem justified. The single death in a 66-year-old patient with advanced pulmonary carcinoma could not be adequately evaluated without autopsy studies and might even be unrelated to the platinum uracil blue. However, it should alert investigators to the possibility of an idiosyncratic cardiac arrest-type reaction.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Bleomycin: NSC-125066
***Cis*-dichlorobiscyclopentylamineplatinum(II):** CAS reg. No. 38780-36-8; PAD
***Cis*-dichlorodiammineplatinum(II):** NSC-119875; CAS reg. No. 15663-27-1
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU
Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamine-6-pteridinyl)methyl]-methylamino]benzoyl]-
6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-
Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Inhibition of Deamination of Arabinosylcytosine (NSC-63878) by Rhodium(II) Acetate [1,2]

S. H. Lee, D. L. Chao, J. L. Bear, and A. P. Kimball [3,4,5]

SUMMARY

The nobel metal "cage" complex, rhodium(II) acetate, was found to inhibit the deamination of the fraudulent nucleoside, arabinosylcytosine. Potent inhibition of a purified cytidine deaminase from mouse kidney was observed when either cytidine or arabinosylcytosine was used as substrate.

[Cancer Chemother Rep 59:661–663, 1975]

It is well known that cellular deaminases compromise the utility of the fraudulent nucleosides, arabinosylcytosine (ara-C) and arabinosyladenine (ara-A),[6] as antitumor agents by converting them to inactive compounds. Potent inhibitors of adenosine and cytidine deaminase would greatly improve the efficacy of ara-A and ara-C in the treatment of neoplasia. Ideally, inhibitors of the deaminases would not be structural analogs of the natural substrates, since substrate analogs might also inhibit the activating kinases.

Efforts have already been made to prevent the rapid deamination of ara-A and ara-C. For instance, Chao and Kimball (1), Lomax and Henderson (2), and Koshiura and LePage (3) have reported successes in the prevention of deamination of ara-A. Ho (4) has utilized the ara-C analog, cyclocytidine,[7] which is resistant to deamination by cytidine deaminase. We report here the prevention of deamination of ara-C by the nobel metal "cage" complex, rhodium(II) acetate, utilizing a purified cytidine deaminase prepared from mouse kidney.

MATERIALS AND METHODS

Cytidine, uridine, and ara-C were purchased from the Sigma Chemical Company, St. Louis, Missouri. Rhodium(II) acetate was purchased from Matthey Bishop, Inc., Malvern, Pennsylvania. Female Swiss mice were obtained from Sprague Dawley, Madison, Wisconsin.

The method of Tomchick et al (5) was used for the isolation and purification of the cytidine deaminase from the kidneys of 200 female Swiss mice. The analytic determination of cytidine deaminase activity was carried out by the method described by Tomchick et al (5). The procedure of Lowry et al (6) was used for protein determinations.

RESULTS AND DISCUSSION

The structure of rhodium(II) acetate is shown in figure 1. The compound contains two rhodium(II) cations liganded equatorially to

[1] Supported by grants CA-12327 and CA-13817 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2] Arabinosylcytosine: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytosine arabinoside.

[3] Departments of Biophysical Sciences and Chemistry, University of Houston, Tex.

[4] Dr. Chao died in Apr 1973.

[5] *Reprint requests to:* Ms. Sang He Lee, Department of Biophysical Sciences, University of Houston, Cullen Blvd, Houston, Tex 77004.

[6] Arabinosyladenine: NSC–404241; CAS reg. No. 5536-17-4; adenine, 9-β-D-arabinofuranosyl-.

[7] Cyclocytidine: NSC-145668; CAS reg. No. 10212-25-6; 6*H*-furo[2′,3′:4,5]oxazolo-[3,2-*a*]pyrimidine-2-methanol, 2,3,3*a*,9*a*-tetrahydro-3-hydroxy-6-imino-, monohydrochloride.

TABLE 1.—Purification of cytidine deaminase

Procedure	Total volume (ml)	Total units	Protein (mg/ml)	Specific activity (nmols/ mg/min)	Relative purity
Whole homogenate	250	15826	32.8	1.9	1
Supernatant	182	9414	22.2	2.3	1.2
Heat denaturation	130	8580	13.2	5.0	2.5
$(NH_4)_2SO_4$ fraction	15	6968	19.0	24.5	12.6
Dialysis (phosphate buffer)	15	6952	16.0	29.0	15.0
Alumina gel C-Gamma	10	4867	10.0	46.8	24.2
Dialysis (acetate buffer)	9	4254	6.7	66.8	35.6

four acetate anions giving this nobel metal "cage" complex a net charge of zero. The axial ligand positions designated *L* in figure 1 lie above and below the plane of the complex. These axial positions are available for ligand formation with electron-donating groups such as ionized carboxyl groups, tertiary nitrogens, sulfurs, and the oxygen of water.

The cytidine deaminase from Swiss mouse kidney was purified 35-fold (table 1). The greatest purification steps occurred from ammonium sulfate precipitation and alumina gel adsorption. This purification was not as good as that reported by Tomchick et al (5), but it allowed us to determine relative substrate specificities and to do inhibition kinetic studies. Mouse kidney cytidine deaminase will deaminate 2'-deoxycytidine and ara-C at about one half and one fourth the rate, respectively, for cytidine (table 2). These data compare quite favorably with those reported by Tomchick et al (5). Rhodium(II) acetate inhibited the cytidine deaminase using ara-C as substrate. There was an increasing inhibition of the enzyme with increasing drug molarities (table 3). The 50% inhibitory molarity of rhodium(II) acetate was found to be 7×10^{-5} M. A Lineweaver-Burk plot for rhodium(II) acetate inhibition of ara-C deamination by cytidine deaminase is shown in figure 2. Least squares analysis was used here to fit the best lines to the data points. The plot showed that rhodium(II) acetate gave an inhibition of the mixed type. This implies that rhodium(II) acetate binds close to but not exactly in the substrate site of the catalytic center of the enzyme. A Hill plot was employed to determine if a critical site(s) (7) on the enzyme was involved in binding of the rhodium (II) acetate (fig 3). We have used this method successfully in our past studies on enzyme-active centers (8–10). The Hill plot in this study has a slope of 1 which shows that one critical site is bound in the vicinity of the catalytic center, and that one molecule of rhodium(II) acetate is bound to one enzyme molecule in that site.

This study shows that rhodium(II) acetate has promise as an inhibitor of the deamination of ara-C, and may possibly be able to increase the efficacy of ara-C as an antitumor agent in man.

TABLE 2.—Substrate specificity of cytidine deaminase

Compounds	Relative rate
Cytidine	100
2'-Deoxycytidine	59
Ara-C	27
Cytosine	0
2'-Deoxycytidine-5'-monophosphate	3
Cytidine-5'-triphosphate	3

TABLE 3.—Inhibition of cytidine deaminase by rhodium (II) acetate

Concentration of rhodium(II) acetate (M)	nmols of ara-C deaminated	Percent of control
0	82.8	100
1×10^{-5}	75.0	91
5×10^{-5}	53.3	64
1×10^{-4}	33.0	40
2.5×10^{-4}	10.0	12
5×10^{-4}	0	0

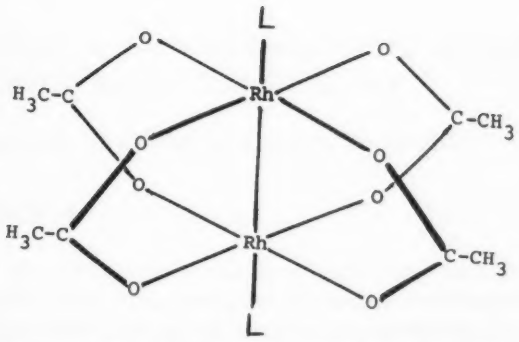

FIGURE 1.—Structure of the rhodium(II) acetate molecule.

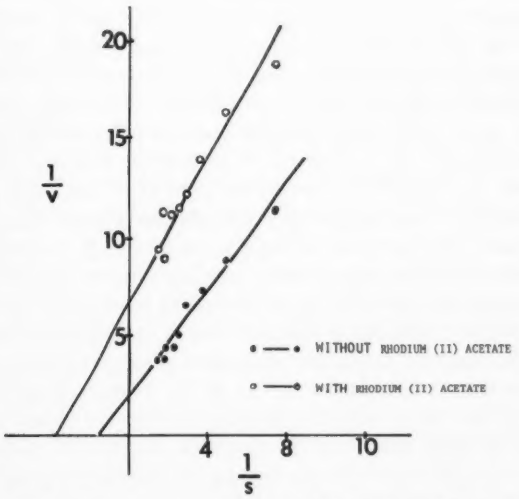

FIGURE 2.—Lineweaver-Burk plot of rhodium(II) acetate inhibition of cytidine deaminase using ara-C as substrate.

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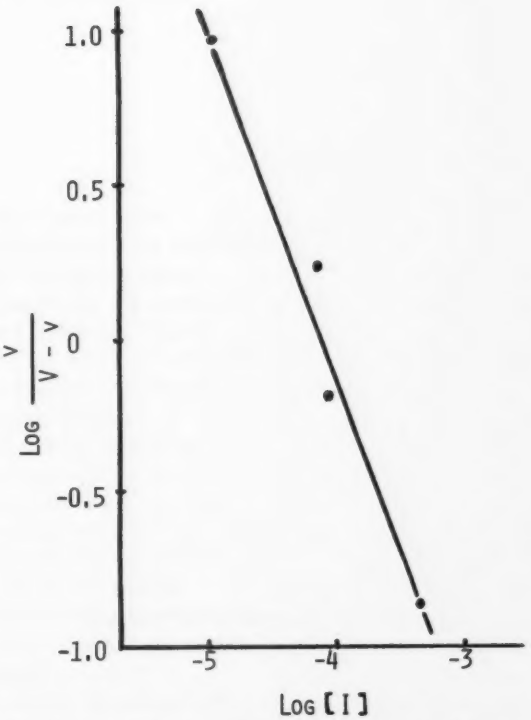

FIGURE 3.—Hill plot of the binding of rhodium(II) acetate to cytidine deaminase.

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Action of *Cis*-dichlorodiammineplatinum (II) (NSC-119875) at the Cellular Level [1,2]

Benjamin Drewinko and Jeffrey A. Gottlieb [3,4]

SUMMARY

Asynchronous human lymphoma cells treated for 1 hour with increasing concentrations of *cis*-dichlorodiammineplatinum (II) revealed a marked decrease in survival as estimated by the colony-forming technique. When the treatment was extended for 8 hours at a concentration of 5 μg/ml, a killing effect (>3 log decades) was observed which was similar to that obtained when 50 μg/ml is incubated with the cells for 1 hour. This finding suggests that better antitumor effects with fewer toxic effects may be obtained clinically by prolonged infusion of low doses of *cis*-dichlorodiammineplatinum (II). Synchronized lymphoma cells showed no significant degree of cell-cycle-stage sensitivity to *cis*-dichlorodiammineplatinum (II). The drug kills cells with similar efficiency in all stages of the cell cycle. No killing effect was elicited after incubation of the cells with spironolactone, a compound said to protect the kidneys from the toxic effects of heavy metals. However, simultaneous incubation of spironolactone and *cis*-dichlorodiammineplatinum (II) did not prevent the lethal action of the second drug. If spironolactone is proven to be an inhibitor of *cis*-dichlorodiammineplatinum (II) nephrotoxicity, it will become a valuable addition to the treatment of human neoplasia with this platinum compound. Lymphoma cells given a "priming" dose of 10 μg/ml of *cis*-dichlorodiammineplatinum (II) failed to repair the induced damage. A second exposure to 10 μg/ml of the drug at various subsequent intervals elicited greater killing effect than that produced by 20 μg/ml given at one time. A clear synergistic effect was noted when *cis*-dichlorodiammineplatinum (II) was given simultaneously with camptothecin or BCNU. The molecular mechanism by which this effect is accomplished is not presently apparent.

[Cancer Chemother Rep 59:665–673, 1975]

Platinum compounds have been shown to be efficacious in the control of neoplastic proliferation in experimental tumors (1–4) and to have some effect in human neoplasia (5–8).

The most active inhibitors of cell proliferation are neutral complexes of *cis*-isomers of

[1] Supported by contracts NO1-CM-61156 and NO1-CM-43801 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. *Cis*-dichlorodiammineplatinum (II) was manufactured by Ben Venue Laboratories, Inc., Bedford, Ohio, and was supplied by Cancer Therapy Evaluation, DCT, NCI.

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platinum (II) and platinum (IV) (9). Clinical use of *cis*-dichlorodiammineplatinum (II) has shown that although effective control can be obtained in certain tumors, toxic effects (especially nephrotoxicity, delayed bone marrow suppression, and audiologic impairment) limit extensive clinical application (10). Nephrotoxicity induced by this drug is similar to that induced by other heavy metals, especially mercury (11). Superior results were obtained when *cis*-dichlorodiammineplatinum (II) was used in combination with cyclophosphamide, isophosphamide, or ICRF-159. Combinations of the platinum compound with camptothecin or BCNU were not more effective than treatment with each drug alone (12).

Although a considerable amount of information is available on the influence of platinum compounds on biomolecular mechanisms and on their pharmacologic properties (13–17), very little is known about the effects of these drugs at the cellular level. The present report analyzes, by using the colony-forming technique, the lethal effects of one such platinum complex, *cis*-dichlorodiammineplatinum (II), in a long-term culture of human lymphoma cells. Dose- and cell-cycle-dependent effects, survival after protracted treatment or as a function of duration of treatment, and effects of simultaneous exposure with other chemotherapeutic agents were analyzed. In addition, the effects of spironolactone used singly or in combination with *cis*-dichlorodiammineplatinum (II) were studied in the same lymphoma cell line. It has been reported that spironolactone protects the kidneys from the toxic effects of heavy metal poisoning (18). The possibility exists that nephrotoxicity will be decreased by using spironolactone in conjunction with the platinum compound if the former drug does not interfere with the killing activity of the latter drug on neoplastic cells.

MATERIALS AND METHODS

Cell Line

An established culture of human immunoglobulin-producing cells (T_1 cells) derived from the involved lymph node of a patient with lymphocytic lymphoma (19) was used as a source for target cells. Monolayer cultures were maintained in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine, and antibiotics. Under these conditions, the average cell-cycle time was 27 hours, the pre-DNA synthesis period (G_1) was 3.5 hours, the DNA synthesis period (S) was 13.5 hours, and the post-DNA synthesis period (G_2) was 10 hours.

Cell Survival Experiments

Experiments involving asynchronous populations were performed on cells in logarithmic growth. Synchronized S-phase cells were obtained by a single treatment with 3 mM thymidine as previously described (20). The degree of synchrony was monitored by 30-minute pulse labeling with 1 microcurie of ^{3}H-thymidine per ml, to determine the percentage of cells in S phase, and by scoring the mitotic index. At the end of the synchrony procedure, 90% of the cells were in S phase and then moved synchronously into G_2 (80%). After a mitotic peak of 11%–14% (the mitotic index of asynchronous cells is 1.6), the cells were still partially synchronized in G_1 (70%–80%). These procedures did not significantly alter the plating efficiency of T_1 cells. In all radioautographic procedures, a 50% solution of Ilford K_5 emulsion in distilled water was used. The radiolabeled cells were exposed for 1–2 weeks and were developed in Kodak D_{19} developer. To assess survival, treated and control cells growing in 60-mm petri dishes were harvested by standard procedures and counted with the aid of an electronic particle counter (Coulter Electronics, Hialeah, Fla). Known aliquots were dispensed into 60-mm petri dishes so that 50–100 colonies would appear after 21 days' incubation in a 5% CO_2 humidified atmosphere at 37°C. The colonies were stained with 0.5% crystal violet in 95% ethanol. Viability was defined as the ability of single cells to give rise to a colony of 50 or more cells. In each experiment, at least six plating-efficiency controls were run in parallel. The survival fractions of the dose points were normalized with respect to the individual controls for each experiment. Percentage of survival was calculated with the aid of a Sigma 5 XDS computer. Each experiment was

repeated at least twice with three dose or time experimental points and six control points each.

Recovery of drug-induced injury was investigated by exposing cells to a full dose in two separate treatments of 15 minutes and the survival was compared to that resulting from cells exposed to the full dose for 15 minutes at one time.

Possible synergistic pair combinations were investigated by the simultaneous exposure to a single concentration of *cis*-dichlorodiammineplatinum (II) and increasing concentrations of other chemotherapeutic agents for 1 hour. The resulting survival curve was compared to the predicted curve obtained from a theoretical additive effect resulting from the survival after exposure to each drug alone.

Drugs

Cis-dichlorodiammineplatinum (II) was obtained as a dry powder. The drug was dissolved in 0.9% NaCl solution and appropriate concentrations were made with fresh medium. The pH of the solution ranged from 7.0 to 7.3. Table 1 lists the other drugs tested, their sources, and the concentrations used.

T_1 cells were also treated with increasing concentrations of spironolactone (Searle and Co., San Juan, Puerto Rico). The 25-mg tablets were pulverized in a mortar and diluted in 25 ml of distilled water obtaining a clear solution which was filtered through a 0.20-μm Nalgene filter (Sybron Corp., Rochester, NY).

RESULTS

Asynchronous T_1 cells incubated for 1 hour with increasing concentrations of *cis*-dichlorodiammineplatinum (II) exhibited an exponential decrease in survival as a function of the concentration of the drug with a mean lethal dose (Do) of 5.5 μg/ml (1 hour). There was no shoulder region in the survival curve (fig 1).

Asynchronous T_1 cells exposed to a single dose of the drug (5 μg/ml) showed an exponential decrement in survival as a function of treatment duration (fig 2). After an 8-hour incubation with *cis*-dichlorodiammineplatinum (II) the survival decreased by more than 3 log decades.

Synchronized cells incubated with either 5 or 10 μg/ml of the drug for 1 hour at various points of the cell cycle demonstrated some fluctuation in survival depending on the stage of the cycle (fig 3). While mid-S-phase and late S-phase cells appeared relatively more resistant, G_2 and especially G_1 cells showed increased sensitivity to the drug. This effect was more evident with the 10-μg/ml concentration, where the

TABLE 1.—Drugs tested with *cis*-dichlorodiammineplatinum (II) against cultured human lymphoma cells

Compound	Source	Increasing concentrations used (μg/ml)
Cis-dichlorodiammineplatinum (II)	NCI	5 or 10
Adriamycin	NCI through the Farmitalia Research Laboratories	0.1,0.25,0.5,0.75,1.0
BCNU	NCI through the Ben Venue Laboratories	10,12,50,75,100
Bleomycin	Bristol Laboratories	10,25,50,75,100
Cytosine arabinoside	The Upjohn Company	100,250,500,750,1000
Cyclocytidine	NCI	25,50,75,100,500
Camptothecin	NCI through the Ben Venue Laboratories	5,10,25,50,75
Prednisolone	NCI	10,25,50,75,100

difference in survival between the more resistant and the more sensitive cells was about fivefold, while such difference was only twofold with the 5-μg/ml concentration. Hence, the platinum drug does not appear to be particularly stage sensitive, at least with clinically relevant concentrations. This fact is further illustrated in figure 4 where cell survival, as a function of the concentration of *cis*-dichlorodiammineplatinum (II) incubated for 1 hour with synchronized cells at selected points of the cell cycle, is shown. The slopes of the survival curves are very similar for G_2, G_1, and mid-S-phase cells (Do = 5, 4, and 6 μg/ml respectively), indicating that the drug kills cells with equal efficiency regardless of the stage of the cell cycle.

T_1 cells incubated for 1 hour with increasing concentrations of spironolactone (5–100 μg/ml) failed to show any decrease in survival. Cells incubated for 1 hour with increasing concentrations of the platinum compound admixed with 50 μg of spironolactone per ml revealed a decrease in survival almost identical to that observed when *cis*-dichlorodiammineplatinum (II) was administered alone (fig 5).

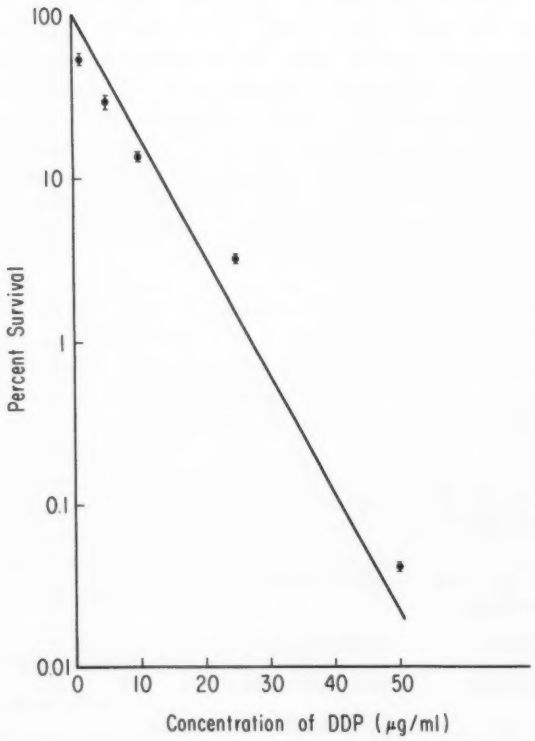

FIGURE 1.—Survival of asynchronous T_1 cells exposed to increasing concentrations of *cis*-dichlorodiammineplatinum (II) for 1 hr. Bars represent standard errors of the mean.

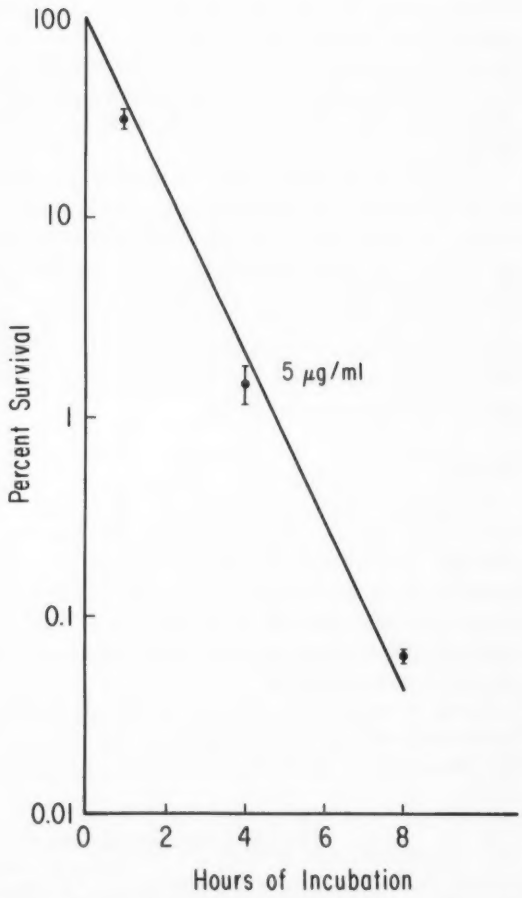

FIGURE 2.—Survival of asynchronous T_1 cells exposed to a single concentration of *cis*-dichlorodiammineplatinum (II) (5 μg/ml) for varying periods of time.

Cells receiving 20 μg/ml of the drug in two separate exposures of 10 μg/ml (15 minutes each) demonstrated no increased survival indicating failure to recover from the "priming" drug-induced damage. In fact, increased killing over that resulting from cells exposed to 20

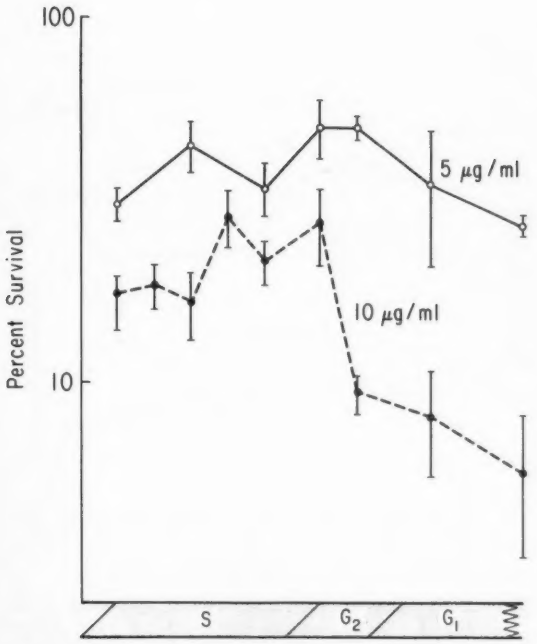

FIGURE 3.—Effect of a single dose of *cis*-dichlorodiammineplatinum (II) on synchronized T_1 cells treated at different points of the cell cycle.

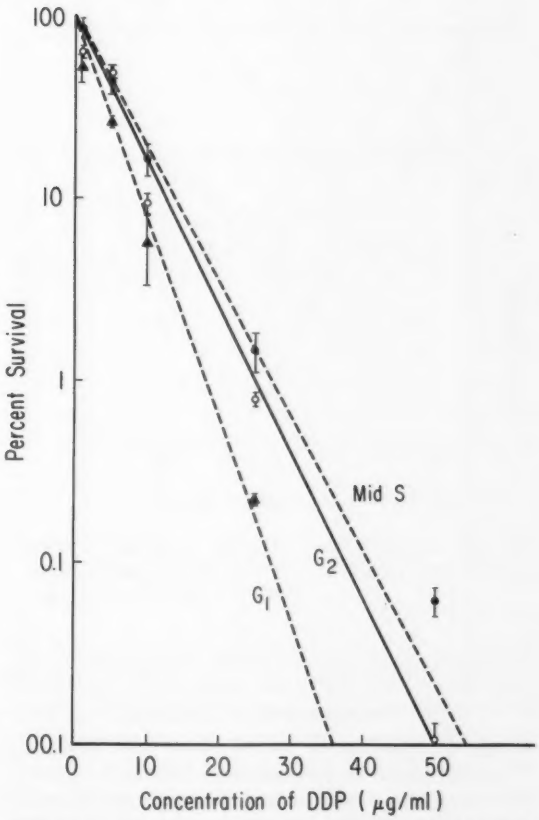

FIGURE 4.—Survival of synchronized T_1 cells treated with increasing concentrations of *cis*-dichlorodiammineplatinum (II) (DDP) at selected stages of the cell cycle.

μg/ml at one time was observed when the interval between treatments exceeded 5 hours, being about 15-fold after 30 hours (fig 6).

Cells were incubated with a concentration of either 5 or 10 μg/ml of *cis*-dichlorodiammineplatinum (II) simultaneously with increasing concentrations of BCNU, bleomycin, adriamycin, cyclocytidine, cytosine arabinoside, camptothecin, and prednisolone. Subadditive to additive effects were noted with bleomycin and cyclocytidine. Additive to moderately supra-additive effects were elicited with adriamycin, prednisolone, and cytosine arabinoside. A tenfold synergistic effect was noted with camptothecin (fig 7) while the shoulder region of BCNU-treated cells was cancelled by simultaneous incubation with the platinum compound (fig 8).

DISCUSSION

Our investigations focused on the effects of *cis*-dichlorodiammineplatinum (II) at the cellular level, as an in vitro model of the lethal activity of the drug *once it reaches the target cells in vivo*. Pharmacologic factors cannot be evaluated with this system, but the data provide information on the time and dose parameters necessary to affect cellular viability, which may guide the development of rational clinical chemotherapeutic protocols.

Our data indicate that the platinum compound is effective in reducing the survival of cultured human lymphoma cells, with a Do of 5.5 μg/ml. Fifty μg/ml can decrease the survivors by more than 3 log decades. However, in the clinical situation, such plasma concentrations may only be reached with doses of 100–

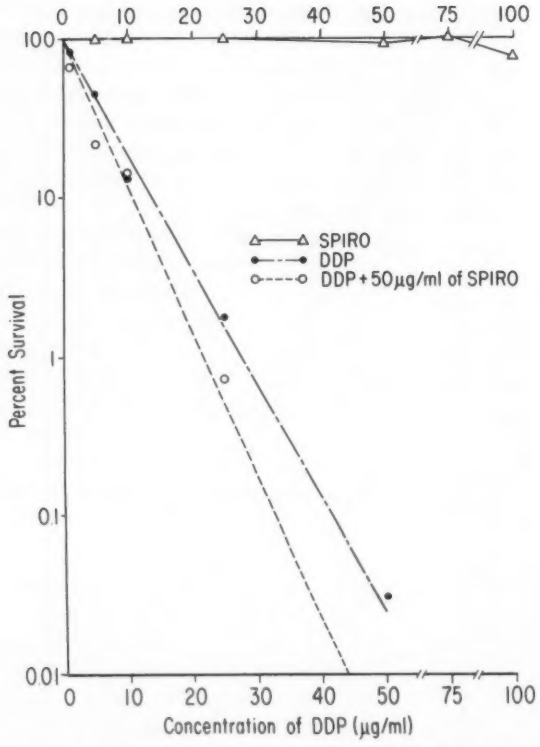

FIGURE 5.—Survival of cells treated with increasing concentrations of spironolactone (SPIRO) and increasing concentrations of *cis*-dichlorodiammineplatinum (II) (DDP) alone and simultaneously with 50 μg/ml of spironolactone.

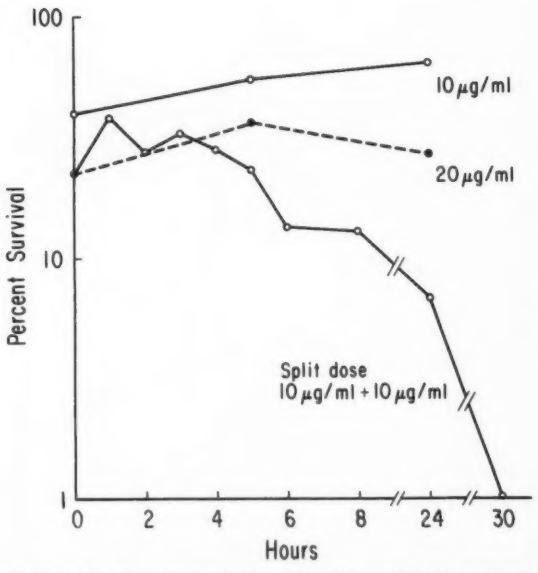

FIGURE 6.—Survival of T_1 cells after split-dose treatment with *cis*-dichlorodiammineplatinum (II). Upper line (open circles) indicates the survival of cells treated with 10 μg/ml for 15 mins at the times indicated by the points. Broken line indicates the survival of cells exposed to 20 μg/ml at the times indicated by the points. Lower line (open circles) presents the survival of cells exposed to 10 μg/ml for 15 mins at one time, washed, reincubated with fresh medium, and re-exposed to an additional 10 μg/ml for 15 mins at the times indicated by the points.

200 mg/m² of *cis*-dichlorodiammineplatinum (II), which may cause considerable toxicity (10,14). At the usual concentrations achieved in the serum of patients (2–5 μg/ml) (C. Stewart, personal communication), only a 50%–70% reduction in cell survival was observed when the lymphoma cells were treated for 1 hour. However, since *cis*-dichlorodiammineplatinum (II) has a low clearance from plasma and, in fact, may accumulate in extracellular fluids (14), a much greater killing effect may be achieved in vivo with such low concentrations. This can be seen in figure 2 which mimics such a clinical situation by exposing the cultured cells to a continuous incubation with 5 μg/ml of the platinum drug. After only 8 hours of continuous incubation, a killing effect (> 3 log decades) is observed, similar to that obtained for cells exposed to 50 μg/ml for only 1 hour. The concentration × time factor is lower for the former modality than for the higher dose short-term treatment. This fact suggests the need to investigate the efficacy of giving low doses of *cis*-dichlorodiammineplatinum (II) in continuous infusion for extended periods of time. Usually, the use of prolonged infusion as a mode of administering a drug is dictated by cell-kinetic properties of the agent, particularly its S-phase sensitivity (21). In the particular case of the platinum drug, this modality would be independent of stage sensitivity but only related to the possibility of obtaining greater antitumor effects with less clinical toxicity. Even though a single bolus injection of *cis*-dichlorodiammineplatinum (II) may produce some ac-

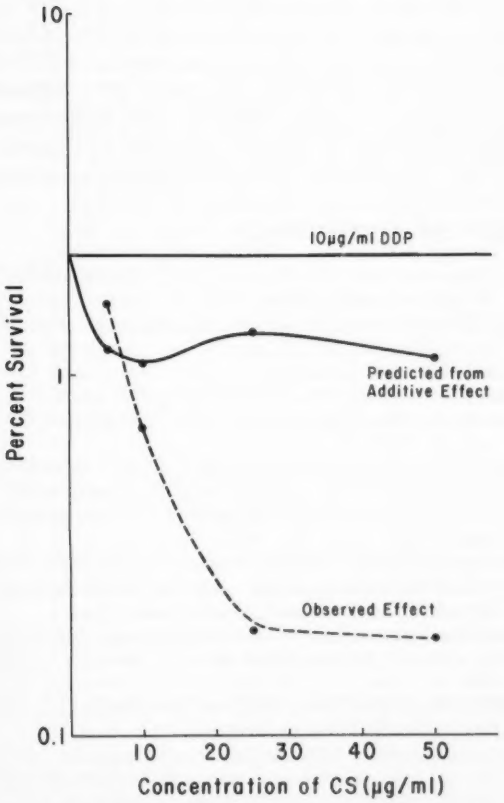

FIGURE 7.—Effect of simultaneous incubation with *cis*-dichlorodiammineplatinum (II) (DDP) and increasing concentrations of camptothecin (CS).

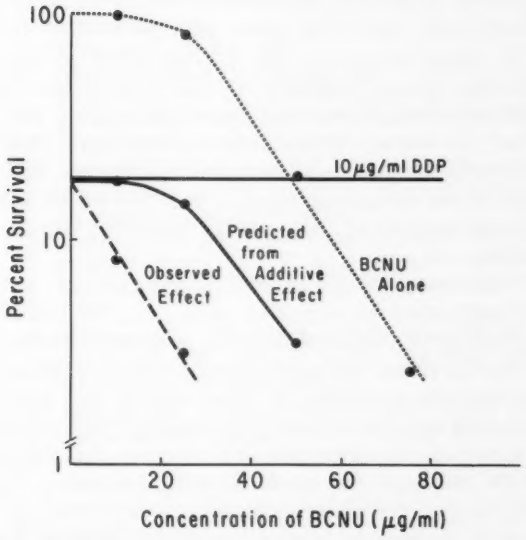

FIGURE 8.—Effect of simultaneous incubation with *cis*-dichlorodiammineplatinum (II) (DDP) and increasing concentrations of BCNU.

cumulation of drug, the dose necessary to achieve an effective concentration in plasma is well beyond the nephrotoxic limit of 1.95 mg/kg (14), and most of the drug is rapidly excreted by the kidneys. A prolonged intravenous infusion of low doses of this agent should permit a steady plasma concentration under controlled conditions.

Synchronized T_1 cells exposed to a single dose of 5 or 10 μg/ml of *cis*-dichlorodiammineplatinum (II) for 1 hour at 2-hour intervals throughout the cell cycle revealed a minor degree of stage sensitivity (fig 3). This was more accentuated with the 10- than with the 5-μg/ml concentration. S-phase cells appeared relatively more resistant than G_1 cells while the sensitivity of G_2 cells was intermediate. Figure 4, depicting the dose-response survival curves of synchronized mid-S, G_2, and G_1 cells, readily demonstrates that there are no significant differential sensitivities to the platinum compound along the cell cycle (Do = 4 μg/ml for G_1; 5 μg/ml for G_2; and 6 μg/ml for S). These data clearly demonstrate that this compound cannot be considered a phase-sensitive drug.

One of the most critical untoward effects of *cis*-dichlorodiammineplatinum (II) is its nephrotoxicity. The changes observed in the kidney are common to those of heavy metal poisoning, especially mercury (11). Recently, Haddow and Lester (18) reported a protective effect of spironolactone in mercury poisoning of rats. These authors considered that spironolactone prevents toxicity by complexing with the heavy metal and increasing its excretion via the biliary tract. If this property of spironolactone extended to all heavy metals, including *cis*-dichlorodiammineplatinum (II), it could be beneficial in chemotherapeutic regimens which use the platinum compound by preventing nephrotoxic effects, provided that spironolactone did not interfere with the lethal activity of *cis*-dichlorodiammineplatinum (II) on the tar-

get neoplastic cells. We explored the latter possibility by incubating the lymphoma cells simultaneously with this agent and spironolactone. No interference of the killing effect of *cis*-dichlorodiammineplatinum (II) was noted. It follows that, if spironolactone does indeed protect the kidneys from platinum toxicity, it may become a valuable adjunct in the therapy with platinum complexes since it does not affect the lethal activity of the drug on the tumor population.

Recovery of platinum-induced damage was investigated by exposing cells to a total dose given in two separate exposures spaced in time. The survivors were compared to those resulting from cells receiving the total dose at one time. If cells are able to repair the damage induced by the drug, the time allowed between exposures will be used for such activity, resulting in greater survival. T_1 cells did not show any capacity to recover from a "priming" damage. In fact, a greater degree of killing was elicited with protracted treatment extended over a period of 30 hours. It has been suggested that the lethal activity of *cis*-dichlorodiammineplatinum (II) is exerted by cross-linking DNA (17,22). Therefore, it is possible that a "priming" dose of the drug may facilitate further crosslinking by providing a partially rigid target site.

Biologic synergism of the platinum compound was investigated by simultaneous incubation with other chemotherapeutic agents. A tenfold increment in lethal effects with *cis*-dichlorodiammineplatinum (II) and camptothecin over that expected from the summation of the effects with each drug alone was noted. It is not apparent how the platinum drug and camptothecin exert their combined effect. It is possible that platinum-induced crosslinking of DNA may facilitate the mode of action of camptothecin, either by inhibiting DNA synthesis by a direct effect on the DNA template (23,24) or by producing strand breaks (25). The shoulder region of the survival curve obtained from BCNU-treated cells was cancelled by *cis*-dichlorodiammineplatinum (II). This indicates that the latter drug affects the capacity of cells to absorb sublethal damage, possibly by modifying the structure of the target sites. Again, the exact molecular mechanism by which this effect is accomplished remains obscure. It is noteworthy that contrary to data obtained in the L1210 leukemia system, both camptothecin and BCNU appeared clearly synergistic with the platinum compound in human cultured cells. Differences may be related to species response, to pharmacologic properties, or to scheduling variations.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8; 14-hydroxydaunorubicin

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Camptothecin: NSC-100880; CAS reg. No. 25387-67-1

***Cis*-dichlorodiammineplatinum (II):** NSC-119875; CAS reg. No. 15663-27-1

Cyclocytidine: NSC-145668; CAS reg. No. 10212-25-6; 6*H*-furo[2',3':4,5]oxazolo-[3,2-*a*]pyrimidine-2-methanol, 2,3,3*a*,9*a*-tetrahydro-3-hydroxy-6-imino-, monohydrochloride

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4'-propylenedi, (±)-

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

Prednisolone: NSC-9900; CAS reg. No. 50-24-8; pregna-1,4-diene-3,20-dione, 11β,17,21-trihydroxy-

Spironolactone: NSC-150399; CAS reg. No. 52-01-7; 17α-pregn-4-ene-21-carboxylic acid, 17-hydroxy-7α-mercapto-3-oxo, γ-lactone, acetate

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ANNOUNCEMENTS

Oncology Placement Service

The American Society of Clinical Oncology's Clearing House of Oncologic Opportunities is accepting inquiries from applicants concerning staff positions and oncology fellowships, as well as announcements of openings for such applicants. The service is open to members and non-members of ASCO. Please direct all inquiries and announcements to Michael J. Brennan, MD, American Society of Clinical Oncology, 110 E Warren, Detroit, Mich 48201.

Eighth World Congress of Gynecology and Obstetrics

Mexico City, October 17–23, 1976

The International Federation of Gynecology and Obstetrics announces the Eighth World Congress of Gynecology and Obstetrics to be held October 17–23, 1976, in Mexico City.

There will be five plenary sessions, seminars on current topics of gynecology and obstetrics, correlated seminars, and three special invited lectures. Within this scientific program there will be free communications sessions, films, and precongress courses as well as scientific, technical, commercial, and artistic exhibits. In addition there will be a social program designed to enable attendants to become better acquainted.

More detailed information about the scientific program and other aspects of the congress will be available in the near future.

Training Program in Medical Oncology

A 2-year training program in Medical Oncology is being offered at Tufts University School of Medicine and affiliated hospitals. Training is shared by the Medical Oncology units of the New England Medical Center, Lemuel Shattuck, and Pondville Hospitals. Elective rotations to Therapeutic Radiology, Surgical Oncology, Pathology, and Hematology are available. Fellowships require an internship and 1 or 2 years of previous residency training. The program is sponsored by the National Cancer Institute and carries a stipend of approximately $13,000 per year.

Further information may be obtained from: Larry Nathanson, MD, Chief, Medical Oncology Service, New England Medical Center Hospital, 171 Harrison Ave, Boston, Mass 02111.

Fourth International Symposium on Senology

June 29–July 3, 1976, Strasbourg, France

The European Association of Radiology announces the Fourth International Symposium on Senology to be held from June 29 to July 3, 1976, in Strasbourg, France.

The subject of the Symposium will be noncancerous diseases of the breast. There will be study groups including invited lectures and discussions concerning (a) investigational methods, (b) mastopathies, and (c) lactation. There will also

be conferences and communications including such topics as breast infections, malformations, plastic surgery, pathology and functions, computer analysis, and comparative psychology. In addition, there will be a scientific and technical exhibit.

The following pre-symposium meetings will be offered: (a) Journees Nationales on Echography (French Society of Echography), June 28–29, 1976, and (b) Post-University Courses on Thermography (European Association of Thermography) and Xerography, June 28, 1976.

Simultaneous translation into English, French, and German will be carried out in all meeting rooms.

For further information write to: Symposium Sein 1976, Professor Charles Gros, Palais des Congres, 67 Strasbourg, France.

Symposium on Steroid Hormone Action and Cancer

Ann Arbor, Michigan, October 27–29, 1975

The Symposium on Steroid Hormone Action and Cancer to be held at the University of Michigan on October 27–29, 1975, is designed to facilitate an exchange of information and ideas between basic and clinical scientists in the areas of steroid hormone action and its relationship to cancer research. It will consist of plenary sessions and round table discussions including such areas as androgens, estrogen, progestins, and corticoids in breast, uterine, and prostatic carcinoma. Participants are encouraged to discuss and share their recent investigative data.

For further information contact: The Office of Intramural Education, The Towsley Center for Continuing Medical Education, University of Michigan, Ann Arbor, Mich 48104.

Medical Oncology Course

The University of Texas System Cancer Center of M. D. Anderson Hospital and Tumor Institute at Houston, in cooperation with The University of Texas Health Science Center at Houston, Division of Continuing Education, presents a Medical Oncology Course to be held on September 24–26, 1975, in Houston. This comprehensive and intensive course is designed to review current concepts of the natural history, diagnosis, therapy, and clinical management of the spectrum of malignant disease cared for by the medical oncologist. It will also review the detection, diagnosis, staging, treatment, and management of malignancies, and will focus on hematologic malignancies, breast cancer, pulmonary cancer, sarcomas, genitourinary cancers, gynecologic cancers, gastrointestinal cancers, and melanomas. The course will consist of 24 hours of didactic lectures with time allotted for discussion. This course has been approved for 24 hours of Category I AMA Physician Recognition Award Credit.

For further information write: The Office of the Director, The University of Texas Health Science Center at Houston, Division of Continuing Education, PO Box 20367, Houston, Tex 77025.

First EORTC Breast Cancer Working Conference

Brussels, Belgium, September 19–20, 1975

The First EORTC Breast Cancer Working Conference, organized by the EORTC Breast Cancer Cooperative Group, will be held September 19–20, 1975, at the Institut Jules Bordet, Brussels, Belgium. Its purpose is to analyze the present status of the various fields of research on breast cancer, both fundamental and clinical. The main topics will be Virology, Immunology, Experimental Models, Endocrine Aspects, Palliative Treatment, Curative Treatment, and Epidemiology and Screening.

Each topic will be presented by European and American investigators followed by a ½ hour round table discussion. At the end of the meeting a consultant committee will be created to draft recommendations and guidelines to serve the EORTC in the coordination and implementation of its research programs.

The official language of the conference will be English. The registration fee is 1000 Belgian francs. The number of participants will be limited to the first 200 registrants. Registrations and inquiries are to be sent to: Dr. M. Rozencweig, Institut Jules Bordet, 1 rue Heger-Bordet, 1000 Brussels, Belgium.

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